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THE ACTION OF RADIUM EMANATION ON THE VITAMINES OF YEAST.

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Since the discovery of the element radium by M. and Mme. Curie in 1898, extensive investigations have been made upon the influence of radium emanation on chemical reactions, animals, plants, and enzymes. A brief review of some of the more characteristic experimental results may be of interest in connection with our present investigation.

Chemical and Physical Effects.

Becquerel[1] has observed that β-rays change yellow phosphorus into red form. Ramsey[2] and Cameron[3] have shown that the radium emanation has destructive and partial synthetic actions upon water, carbon monoxide, carbon dioxide, hydrogen chloride, and ammonia. The decomposing action of radium rays on the lecithin molecule has been observed by Schwarz,[4] Schaper,[5] and Werner.[6] On the other hand, no specific destructive effect was found by Thies[7] and Hertwig.[8] Hardy[9] has reported that radium emanation has the power of dissolving and coagulating globulin in acid and alkaline solutions respectively. Dreyer and Hanssen[10] have shown that the rays of radium coagulate vitellin; but seemingly it has no effect upon albumin.

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[1] Becquerel, H., *Compt. rend. Acad.*, 1901, cxxxiii, 709.

[2] Ramsey, W., *J. Chem. Soc.*, 1907, xci, 931.

[3] Cameron, A. T., and Ramsey, W., *J. Chem. Soc.*, 1907, xci, 1593; 1908, xciii, 966.

[4] Schwarz, G., *Arch. Physiol.*, 1903, c, 532.

[5] Schaper, A., *Deutsch. med. Woch.*, 1904, xxx, 1434.

[6] Werner, R., *Deutsch. med. Woch.*, 1905, xxxi, 61, 1072.

[7] Thies, A., *Mitt. Grenzgeb. Med. u. Chir.*, 1905, xiv, 694.

[8] Hertwig, O., *Deutsch. med. Woch.*, 1911, xxxvii, 2209.

[9] Hardy, W. B., *J. Physiol.*, 1903, xxix, p. xxix.

[10] Dreyer, G., and Hanssen, O., *Compt. rend. Acad.*, 1907, cxlv, 234.

Animals.

Giesel[11] and Becquerel and Curie[12] have observed that when skin was exposed to radium emanation it was burned. Abbe[13] has shown that radium emanation has retrograde action on morbid cell growths (ordinary wart and epithelial cancer of the ear). Prime[14] has shown that β- and γ-rays do not kill the cells outright, but injure the nucleus in such a manner as to prevent the further division, which must eventually result in the death of the cell. A recent report of Janeway[15] showed very clearly the successful treatment of a number of cancer patients by means of radium emanation. Ewing[16] has observed that γ-rays of radium are capable of causing definite regressive changes in deep seated tumors (mediastinal lymphosarcoma, carcinoma of the lungs, and abdominal metastases of carcinoma of the testis). Tur[17] has studied the influence of radium rays upon the chick embryos and showed monstrous embryos were produced. Willcock[18] has investigated the influence of radium rays upon protozoa and found that *Hydra fusca* and *Actinosphaerium* did not respond to the rays of radium and were killed. *Hydra viridis*, *Nyctotherus*, *Balantidium*, *Opalina*, and *Euglena* on the other hand responded to the rays and showed no signs of injury. This latter writer has also pointed out that only those protozoa which contain chlorophyll respond to the rays of radium. Bohn[19] has observed that spermatozoids of sea urchins are enfeebled and killed by radium rays; but their eggs are more readily fecundated.

Plants.

Giesel[11,20] observed the effect of radium rays upon the chlorophyll in leaves, noting that the treated leaves turned yellow and withered away. Dixon[21] exposed seeds to a tube containing 5 mg. of radium bromide. He found that seedlings immediately under the tube were retarded in their growth. Extensive and interesting investigation on the influence of radium emanation upon the growth of plants has been made by Gager.[22] He showed that the radium emanation under certain conditions acted as a stimulus both to the germination of the seeds and the growth of the plant.

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[11] Giesel, F., *Ber. chem. Ges.*, 1900, xxxiii, 3569.
[12] Becquerel, H., and Curie, P., *Compt. rend. Acad.*, 1901, cxxxii, 1289.
[13] Abbe, R., *Med. Rec.*, 1904, lxvi, 321.
[14] Prime, F., *J. Cancer Research*, 1917, ii, 107.
[15] Janeway, H. H., *Surg., Gynec. and Obst.*, 1918, xxvi, 233.
[16] Ewing, J., *J. Cancer Research*, 1919, iv, 45.
[17] Tur, J., *Compt. rend. Soc. biol.*, 1904, lvii, 236.
[18] Willcock, E. G., *J. Physiol.*, 1904, xxx, 449.
[19] Bohn, G., *Compt. rend. Acad.*, 1903, cxxxvi, 1085.
[20] Giesel, F., *Samml. chem. u. chem.-tech. Vortr.*, 1902, vii, 1.
[21] Dixon, H. H., *Nature*, 1903, lxix, 5.
[22] Gager, C. S., *Memoirs N. Y. Botanical Garden*, 1908, iv.

On the other hand, it has retarding and inhibitory action if prolonged exposure or greater activity of radium was applied. He also showed that the β-rays are physiologically more effective than γ-rays. Aschkinass and Caspari[23] showed that the growth of *Bacillus prodigiosus* on agar culture medium was retarded by the action of radium rays. Pfeiffer and Friedberger[24] have observed that the exposure of radium rays from 25 mg. of radium bromide at a distance of 1 cm. has inhibitory action upon the growth of *Bacillus typhosus* and *Spirillum choleræ*. Crookes[25] has reported that the radium emanations or in other words "electrons" have destructive action upon *Bacillus liquefaciens*, *Bacillus coli communis*, and *Bacillus prodigiosus*. Dixon and Wigham[26] found that the β-rays from radium bromide exercise marked inhibitory action on the growth of *Bacillus pyocyaneus*, *Bacillus typhosus*, *Bacillus prodigiosus*, and *Bacillus anthracis* in agar culture medium. Van Buren and Zinsser[27] have shown that radium emanation has no inhibitory action upon the growth of *Bacillus typhosus* and *Staphylococcus pyogenes aureus;* but has distinct, though limited, inhibitory action on *Bacillus pyocyaneus*. Prescott[28] has shown that radium rays, activity of 1,500,000, have no effect upon the fresh cultures of *Bacillus coli*, *Bacillus diphtheriæ*, or *Saccharomyces cerevisiæ* at a distance of 1 cm. when the time exposure is less than 90 minutes.

Enzymes.

Henri and Mayer[29] have shown that the activities of invertase, emulsin, and trypsin are very much destroyed through prolonged exposure to radium rays. Schmidt-Nielsen[30] showed that the radium preparation of 1,800,000 activity has slight inhibitory action upon rennin. Willcock[31] has reported that the radium rays are injurious to digestive ferments such as pepsin, trypsin, and ptyalin. Brown[32] has investigated the influence of radium upon pepsin, pancreatic diastase, and autolytic ferment of the dog's liver. He found that the very radio-active radium D, radium E, and radium F have marked inhibitory action upon pepsin and diastase; but have no effect upon the autolytic ferment.

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[23] Aschkinass, E., and Caspari, W., *Arch. ges. Physiol.*, 1901, lxxxvi, 603.
[24] Pfeiffer, R., and Friedberger, E., *Berl. klin. Woch.*, 1903, xl, 640.
[25] Crookes, H., *Chem. News*, 1903, lxxxvii, 308.
[26] Dixon, H. H., and Wigham, J. T., *Nature*, 1903, lxix, 81.
[27] Van Buren, F., Jr., and Zinsser, H., *Am. Med.*, 1903, vi, 1021.
[28] Prescott, S. C., *Science*, 1904, xx, 246.
[29] Henri, V., and Mayer, A., *Compt. rend. Acad.*, 1904, cxxxviii, 521.
[30] Schmidt-Nielsen, S., *Mitt. Finsens med. Lysinst. in Kopenhagen*, 1906, x, 107.
[31] Willcock, E. G., *J. Physiol.*, 1906, xxxiv, 207.
[32] Brown, T. R., *Arch. Int. Med.*, 1912, x, 405.

Marshall and Rowntree's[33] investigation showed that the radium emanation has no accelerating influence upon the lipase of the pig's liver or castor oil bean, while inhibition of the enzymatic activity is suggested. Neuberg[34] has shown that radium rays have accelerating action on autolytic ferments. Bergell and Bickel[35] have observed that the activity of pepsin is enhanced by the influence of radium rays. Influence of radium emanation upon autolysis of normal and pathological tissues has been studied by Löwenthal and Edelstein.[36] They found that the rate of increase in autolysis varied with the character of material allowed to autolyze; but greatest accelerating influence was found in cases of human carcinoma tissue. Schulz[37] observed that the radium emanation has a certain amount of accelerating action upon the uric acid-forming ferments of the spleen. Gager[22] has shown that the alcoholic fermentation by Fleischmann's yeast was accelerated when 1 gm. of yeast was pressed closely around a glass tube containing radium bromide of 1,500,000 activity for 20 minutes. Harris and Creighton[38] have reported that radiations from 5 mg. of radium bromide had no appreciable influence on the activity of reductase of the cat's liver.

Searching for a method to differentiate beri-beri vitamine and that of growth-promoting factors in yeast, Funk[39] has investigated the influence of radium emanation on the yeast vitamines. He reported that the radium emanation has no destructive action on beri-beri vitamine. Funk also stated that the radium emanation possesses no action on the growth-promoting factors in yeast. Unfortunately he did not indicate the quantity of the radium emanation used or the time of its action upon the yeast.

The mechanism of the destructive effects of γ-rays upon various neoplasms remains wholly unexplained. In view of the fact that these neoplasms are much more susceptive to the rays than is normal tissue it would seem possible that the growth-promoting vitamines are affected by the rays. The present investigation was planned to answer this question as definitely as possible. Yeast was adopted as the vitamine-containing material, and portions of yeast subjected to the γ-rays of radium were tested for their growth-promoting powers upon young white rats, as compared with the same yeast not treated with radium. In

[33] Marshall, E. K., Jr., and Rowntree, L. G., *J. Biol. Chem.*, 1913–14, xvi, 379.

[34] Neuberg, C., *Verhandl. deutsch. path. Ges.*, 1904, vii, 157.

[35] Bergell, P., and Bickel, A., *Verhandl. Cong. inn. Med.*, 1905, xx, 157.

[36] Löwenthal, S., and Edelstein, E., *Biochem. Z.*, 1908, xiv, 484.

[37] Schulz, A., *Biochem. Z.*, 1913, xlviii, 86.

[38] Harris, D. F., and Creighton, H. J. M., *J. Biol. Chem.*, 1915, xxi, 303.

[39] Funk, C., *Proc. Soc. Exp. Biol. and Med.*, 1916–17, xiv, 9.

dealing with a substance so rich in vitamines as yeast it is necessary to take certain precautions to guard against error in the interpretation of results. Thus it is quite possible that although a considerable percentage of the vitamine may have been destroyed by the radium, a sufficient amount may remain uninjured to give normal growth where an excess of the yeast is added to the diet. The first experiments detailed below show that this is true. We have therefore included a determination of the minimal yeast content necessary for normal growth.

Young albino rats were fed upon a modified artificial diet of Funk and Macallum[40] consisting of casein, cane sugar, starch, butter fat, agar, salt mixture, and radium-treated or untreated yeast preparations. The methods of the purification of commercial washed casein and of the preparations of the yeast and the salt mixture are the same as those mentioned in a previous paper.[41] Corn-starch was purified like casein.

The radium emanation was collected in a capillary glass tube and sealed. These capillary emanation tubes are less than 0.5 mm. in outside diameter and from 12 to 20 mm. in length. The quantity of radium emanation (we used only the γ-rays) was measured by means of an electrometer by the γ-ray method. Since emanation tubes are thin and easy to break they were placed in a glass tube having a diameter of about 5 mm. and thickness of about 0.5 mm.

Our incomplete artificial diet consisted of casein 22 per cent, cane sugar 10 per cent, starch 33 per cent, butter fat 30 per cent, agar 2 per cent, and salt mixture 3 per cent and this was designated as Ration A. Ration B is the same as Ration A except that 6 per cent of yeast preparation replaced 6 per cent of the starch.

A few separate experiments with Ration A showed that young vigorous albino rats either maintained body weight or showed a slight decline in weight in about 7 days; while with Ration B animals gained on the average 1.2 to 3.5 gm. daily during the experimental periods of from 15 to 30 days.

Experiment 1. Action of Radium Emanation on Dry Yeast Preparation.—25 gm. of finely powdered yeast preparation in a small glass bottle were allowed to remain in contact with a glass tube containing 210 millicuries

[40] Funk, C., and Macallum, A. B., *J. Biol. Chem.*, 1916, xxvii, 51.
[41] Sugiura, K., and Benedict, S. R., *J. Biol. Chem.*, 1918, xxxvi, 172.

at the initial hour for 5 days at room temperature with occasional shaking. 6 per cent of yeast preparation in Ration B was replaced by this radium-treated yeast preparation and this special diet was designated as Ration C.

Table I shows the results obtained from feeding animals with Rations A, B, and C.

TABLE I.

Diet.		Day.	♂ 88	♀ 89
	per cent		*gm.*	*gm.*
Normal		0	129.9	98.9
Ration A:		1	136.3	102.6
Casein	22	2	133.8	103.8
Cane sugar	10	3	127.2	101.4
Starch	33	4	123.2	99.7
Butter fat	30	5	125.6	98.5
Agar	2			
Salt mixture	3			
Ration B:		6	129.4	98.9
Casein	22	7	132.3	100.4
Cane sugar	10	8	134.0	103.0
Starch	27			
Butter fat	30			
Agar	2			
Salt mixture	3			
Yeast	6			
Ration C:		9	136.1	105.8
Casein	22	10	137.6	107.1
Cane sugar	10	11	139.0	109.2
Starch	27	12	142.1	110.0
Butter fat	30	13	144.3	111.2
Agar	2	14	145.6	113.1
Salt mixture	3	15	147.9	114.8
Yeast (radium-treated)	6			

Experiment 2. Action of Radium Emanation on Wet Yeast Preparation.—30 gm. of finely powdered yeast preparation were made into a paste with 50 cc. of distilled water. The wet yeast preparation in a small glass bottle was allowed to remain in contact with an emanation tube containing 170 millicuries at the initial hour for 48 hours at room temperature with occasional stirring. 6 per cent of yeast preparation in Ration B was replaced by the same quantity of this radium-treated yeast preparation and this diet was called Ration D.

The comparative results with Rations B and D are given in Tables II and III.

TABLE II.

Diet.	Day.	♀ 210	♂ 211	♀ 212	♀ 213	♀ 214	♂ 215
per cent		*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Normal	0	70.8	44.9	36.1	55.0	43.0	74.0
Ration D:	1	80.9	48.8	43.0	61.1	48.1	80.0
Casein 22	2	81.0	50.4	43.9	63.4	50.6	81.3
Cane sugar 10	3	77.9	52.3	44.7	60.0	52.9	83.4
Starch 27	4	80.1	56.5	46.6	69.7	56.5	87.0
Butter fat 30	5	83.0	58.6	48.6	70.6	59.5	90.6
Agar 2	6	84.8	64.8	53.1	72.5	62.9	93.4
Salt mixture 3	7	84.5	66.5	55.4	72.2	66.9	94.3
Yeast (radium-treated) 6	8	87.3	69.4	56.3	75.6	70.0	96.8
	9	88.5	73.1	58.7	79.6	74.1	100.9

TABLE III.

Diet.	Day.	♂ 216	♀ 217	♂ 218	♀ 219	♀ 220	♀ 221
per cent		*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Normal	0	42.5	34.2	36.8	44.5	35.4	91.4
Ration B:	1	46.4	39.5	43.4	48.7	40.4	96.0
Casein 22	2	47.4	39.7	45.7	50.2	41.4	95.4
Cane sugar 10	3	48.3	38.3	49.0	52.2	42.5	94.2
Starch 27	4	50.0	40.7	50.8	55.6	44.2	96.0
Butter fat 30	5	53.3	42.4	53.2	59.8	46.4	96.8
Agar 2	6	55.7	45.4	57.3	63.6	49.9	97.5
Salt mixture 3	7	58.2	47.3	60.8	66.1	52.0	96.0
Yeast 6	8	60.4	49.1	65.2	68.6	56.7	97.4
	9	64.3	51.4	68.8	72.4	60.0	100.5

It is a known fact that any surface which is exposed to the radium emanation will be coated with a very thin film of Ra-A and its subsequent products.

Experiment 3. Influence of Radio-Active Deposit.—9 gm. of finely powdered yeast preparation were placed in a small glass bulb having a capillary tube at one end. Radium emanation was introduced into this bulb and sealed at the capillary end. It contained 89 millicuries. The mixture was allowed to stand for 96 hours at room temperature with frequent shaking. At the end of the period the capillary tube was opened and the emanation

allowed to escape. 6 per cent of yeast preparation in Ration B was replaced with an equal quantity of this radium-treated yeast preparation and was designated as Ration E.

Table IV shows the results obtained with this special diet.

TABLE IV.

Diet.		Day.	♂ 216	♀ 219	♀ 220	♂ 229
	per cent		*gm.*	*gm.*	*gm.*	*gm.*
Ration A		0	97.6	101.4	94.6	79.6
Ration E:		1	98.4	103.0	96.0	80.7
Casein	22	2	99.3	104.1	97.0	83.6
Cane sugar	10	3	101.5	105.3	98.0	85.0
Starch	27	4	105.8	108.8	100.7	88.0
Butter fat	30	5	108.1	110.2	102.2	89.7
Agar	2	6	110.0	111.5	103.8	92.0
Salt mixture	3					
Yeast (radium-treated)	6					

From Experiments 1 to 4 it is plain that artificial diets containing 6 per cent of radium-treated yeast preparation give the normal expected growth of young albino rats, within the limits of experimental error.

Many of the investigators, who prepare a complete artificial diet, nearly similar to our diet in its composition and constituents, use 6 per cent of yeast; while others use a smaller amount of yeast.

Our second investigation was to find the minimum quantity of yeast preparation needed in order to make a complete diet when added to an incomplete artificial diet.

The results shown in Table V were obtained from a large number of young albino rats fed at first upon Ration A for about 7 days in order to establish an equilibrium in body weight; and which were then fed upon the special diets, either in series or in parallel, consisting of 0.1, 1.0, 2.0, or 6.0 per cent of our yeast preparation and together with all other constituents of Ration A, except that variations in the amounts of yeast preparation were made up by varying the amount of starch.

Table V shows that 2 per cent of yeast content in our artificial diet is sufficient to promote normal growth in young animals.

This diet was called Ration F, and the diet which contained 1.0 per cent of yeast preparation was called Ration G.

TABLE V.

Average Daily Gain in Body Weight of a Young Albino Rat Fed upon an Artificial Diet Containing Different Amounts of Yeast.

0.1 per cent.	1.0 per cent.	2.0 per cent.	6.0 per cent.
gm.	*gm.*	*gm.*	*gm.*
0.15	0.74	2.04	2.17

Experiment 4. Action of Radium Emanation on Dry Yeast Preparation.—25 gm. of finely powdered yeast preparation in a small glass bottle were allowed to remain in contact with a glass tube containing emanation tubes for 36 days at room temperature with occasional shaking. At first the quantity of emanation was 150 millicuries, on the 13th day 83 millicuries, and on the 26th day 99 millicuries were added. Therefore at the end of this experiment there were approximately 44,000 millicuries hours in total. 6 per cent of yeast preparation in Ration B was replaced by 2 per cent of this radium-treated yeast preparation and the difference was made up by starch; this diet was called Ration H. One containing 1 per cent of this radium-treated yeast was called Ration I.

The comparative results in series obtained from these diets are presented in Table VI.

From Table VI we obtain the following results: The average daily gain in the body weight of rats was 1.22 and 1.30 gm. when fed on Rations F and G respectively; while with Rations H and I (containing radium-treated yeast) the average gain was 0.75 and 0.36 gm. respectively.

Experiment 5. Action of Radium Emanation on a Complete Artificial Diet.—210 gm. of Ration F were well mixed and placed in a small porcelain dish. A glass tube containing 160 millicuries of radium emanation was placed in the center of the diet and allowed to stand in a refrigerator for 7 days with frequent mixing. This mixture was kept in a refrigerator together with the remaining emanation until used. We call this diet Ration J and results with this diet are shown in Table VII.

Experiment 6. Action of Radium Emanation on Dry Yeast Preparation.—15 gm. of finely powdered yeast preparation in a small glass bottle were allowed to remain in contact with emanation tubes for 40 days at room temperature with occasional shaking. In the beginning we placed emanation tubes containing 105 millicuries into the yeast. On the 9th day a glass sphere, 4 mm. in diameter, was added which contained emanation of

TABLE VI.

Diet.		Day	♂ 273	♀ 274	♀ 275	♀ 276	♀ 277	♀ 278	♀ 279	♂ 280	♀ 281
	per cent		*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Normal		0	38.6	42.4	34.6	42.8	40.5	36.3	83.3	101.7	76.1
Ration A:		1	44.7	43.9	40.9	47.1	43.1	42.5	80.0	100.0	76.7
Casein	22	2	43.0	42.9	39.3	44.7	41.7	42.6	79.5	99.5	78.8
Cane sugar	10	3	41.7	43.9	39.8	45.4	41.2	42.5	81.5	102.3	79.7
Starch	33	4	44.0	43.9	40.7	45.3	42.7	42.5	82.0	106.1	83.0
Butter fat	30										
Agar	2										
Salt mixture	3										
Ration G:		5	43.1	46.6	41.9	46.8	44.3	44.5	83.2	105.5	82.5
Casein	22	6	44.4	48.1	43.1	47.2	46.5	45.6	85.2	109.2	85.3
Cane sugar	10	7	44.5	49.9	44.3	47.5	47.4	44.5	84.8	110.7	84.7
Starch	32	8	45.0	52.4	45.4	48.3	48.8	47.1	85.0	113.5	86.9
Butter fat	30	9	45.7	54.3	48.0	49.5	50.2	48.4	86.3	116.1	90.0
Agar	2										
Salt mixture	3										
Yeast	1										
Ration I:		10	44.3	53.5	46.8	46.4	50.9	47.4	88.3	117.8	91.1
Casein	22	11	45.0	54.7	47.7	47.2	51.4	48.5	88.3	120.3	92.8
Cane sugar	10	12	44.7	53.4	47.4	47.7	50.7	47.9	87.7	122.0	91.6
Starch	32	13	45.1	53.4	48.1	48.1	51.2	48.4	88.6	123.0	93.1
Butter fat	30	14	45.2	53.4	48.2	49.3	53.2	49.6	88.6	123.9	93.1
Agar	2										
Salt mixture	3										
Yeast (radium-treated)	1										
Ration H:		15	45.7	55.3	47.7	49.6	52.3	49.0	88.6	122.7	93.2
Casein	22	16	46.2	56.4	50.2	51.2	53.4	50.7	87.2	125.6	92.0
Cane sugar	10	17	47.4	59.0	50.4	50.2	55.4	52.6	87.5	127.4	95.2
Starch	31	18	46.9	59.7	50.6	51.3	55.9	52.1	89.9	126.5	93.4
Butter fat	30	19	48.0	60.8	51.3	51.2	56.9	53.2	91.2	129.4	96.5
Agar	2										
Salt mixture	3										
Yeast (radium-treated)	2										
Ration F:		20	49.7	62.9	52.0	52.9	56.5	53.3	92.9	130.0	97.0
Casein	22	21	50.9	64.0	54.7	54.3	57.1	55.7	97.5	134.2	99.3
Cane sugar	10	22	51.0	64.6	54.9	56.1	58.0	57.1	98.6	135.0	96.8
Starch	31	23	51.6	66.0	55.1	55.5	59.5	57.6	94.3	136.4	99.0
Butter fat	30	24	52.6	68.2	55.6	57.4	60.9	58.8	95.8	139.2	101.1
Agar	2	25	53.0	69.6	57.0	57.3	63.0	60.9	96.8	139.6	102.7
Salt mixture	3	26	54.0	69.7	58.4	59.1	62.4	61.2	96.8	140.8	103.3
Yeast	2	27	55.9	70.5	59.5	60.0	63.8	62.2	98.4	144.2	104.0
		28	56.2	72.8	62.1	60.8	65.1	63.2	98.9	146.9	104.7
		29	58.4	74.4	64.0	62.6	67.2	64.5	101.5	149.7	105.8

TABLE VII.

Diet.			Day.	♂ 235	♀ 234	♀ 228
	per cent			*gm.*	*gm.*	*gm.*
Normal			0	89.5	87.0	93.7
Ration A:			1	87.8	86.0	94.2
Casein	22		2	89.4	85.4	91.9
Cane sugar	10		3	88.1	85.7	92.0
Starch	33		4	88.3	86.0	91.4
Butter fat	30		5	88.2	86.3	90.9
Agar	2					
Salt mixture	3					
Ration J:			6	88.6	89.8	92.2
Casein	22		7	91.1	90.7	91.7
Cane sugar	10		8	91.9	91.1	91.8
Starch	31	Radium-	9	89.9	88.4	87.7
Butter fat	30	treated.	10	91.1	90.5	88.4
Agar	2		11	93.4	90.4	87.5
Salt mixture	3					
Yeast	2					
Ration F:			12	98.4	95.0	89.9
Casein	22		13	100.1	96.4	91.9
Cane sugar	10		14	101.8	97.1	91.7
Starch	31		15	103.4	98.3	89.5
Butter fat	30		16	105.7	100.3	91.9
Agar	2					
Salt mixture	3					
Yeast	2					

150 millicuries. On the 15th day we added 81 millicuries and on the 26th day removed all old emanation tubes from the bottle and added new tubes containing 105 millicuries. Therefore at the end of this experiment there were approximately 64,000 millicuries hours in total. 2 per cent of yeast preparation in Ration F was replaced by the same amount of this radium-treated yeast preparation and this was called Ration K.

The comparative results in series obtained from these diets are presented in Table VIII.

A graphic comparison of the average daily gain in the body weight of rats induced from eating Rations A, F, and K is shown in Chart 1.

From Table VIII we obtain the following results: The average daily gain in the body weight of rats was 1.37 gm. when fed on

TABLE VIII.

Diet.		Day.	♂ 306	♀ 313	♀ 340	♀ 341	♂ 342	♂ 343
	per cent		*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Normal		0	41.7	41.5	66.3	53.8	39.0	32.6
Ration A:		1	40.9	43.5	66.0	41.0	40.2	33.1
Casein	22	2	40.0	40.0	66.1	52.5	39.0	33.2
Cane sugar	10	3	39.3	39.7	66.0	53.2	38.9	32.3
Starch	33	4	39.4	38.5	66.1	53.2	39.5	33.7
Butter fat	30							
Agar	2							
Salt mixture	3							
Ration K:		5	37.4	37.3	64.6	50.0	39.2	31.6
Casein	22	6	38.2	40.0	65.0	52.3	39.7	28.3
Cane sugar	10	7	40.7	40.7	65.7	52.8	40.2	27.1
Starch	31	8	40.5	40.7	63.5	53.8	41.2	29.6
Butter fat	30	9	38.7	40.8	64.0	53.3	40.4	31.2
Agar	2	10	42.7	43.2	67.9	56.1	41.6	31.7
Salt mixture	3	11	42.3	43.8	69.4	56.1	42.2	32.0
Yeast (radium-treated)	2	12	44.2	44.3	69.5	59.2	44.2	31.4
		13	42.2	42.2	66.8	58.8	44.6	31.5
		14	43.1	45.1	68.2	59.2	45.0	32.1
Ration F:		15	47.2	47.1	70.5	60.5	46.2	32.6
Casein	22	16	48.0	48.5	73.0	62.7	48.6	34.8
Cane sugar	10	17	50.5	49.0	74.7	65.4	50.0	35.0
Starch	31	18	50.0	50.2	75.6	65.8	53.0	36.3
Butter fat	30	19	52.1	52.3	76.5	68.6	55.0	36.9
Agar	2	20	54.4	54.0	79.2	69.4	56.3	39.7
Salt mixture	3	21	57.8	54.5	78.4	70.3	57.9	39.6
Yeast	2	22	57.6	53.2	79.4	71.8	58.7	38.6
		23	58.6	55.4	79.1	74.3	60.0	40.4
		24	60.0	55.6	80.0	75.2	61.6	42.0
Ration K:		25	61.6	54.7	79.2	74.2	59.2	42.4
		26	63.3	56.3	82.4	78.5	55.3	43.0
		27	63.3	55.7	83.7	78.9	52.4	43.6
		28	65.0	56.0	82.8	78.8	50.0	44.3
		29	67.1	55.5	85.3	82.0	45.8	45.4
		30	66.8	57.2	85.9	82.8	*	46.3
		31	66.8	56.0	85.7	84.2		46.7
		32	70.0	56.4	87.0	85.0		47.3
		33	71.0	54.4	87.6	85.2		44.1
		34	71.0	50.5	89.0	84.8		42.1

* Died.

Ration F; while with Ration K (containing radium-treated yeast) the average gain was 0.38 gm. in the first period and 0.41 gm. in the second period.

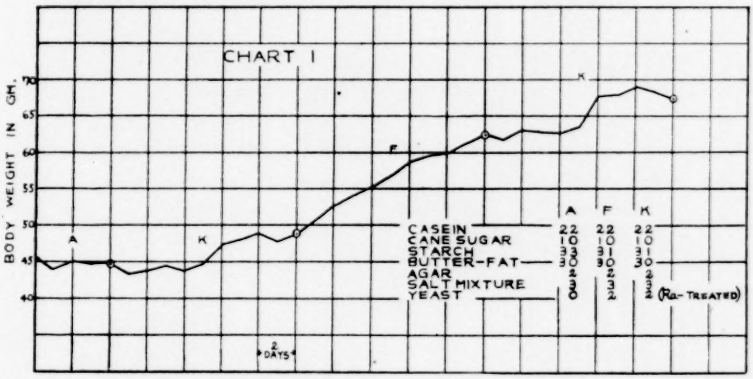

CHART 1.

CONCLUSIONS.

1. Growth-promoting factors in yeast may be inactivated partially by means of exposure to radium emanation. It is possible that the therapeutic effect of radium upon neoplasms may be due in part, at least, to this destruction of growth-promoting substance.

2. 2 per cent of yeast content in our purified artificial diet seems to be sufficient to promote normal growth in young albino rats.

The writers wish to express their great appreciation to the members of the Radium Department, Memorial Hospital, for supplying us with emanation tubes.

BIOCHEMICAL CHANGES IN THE MUSCLE TISSUE OF KING SALMON DURING THE FAST OF SPAWNING MIGRATION.*

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INTRODUCTORY.

Quantitative chemical studies are presented showing the fasting changes in the king salmon, *Oncorhynchus tschawytscha*. This largest and finest food fish among the salmonoids has a biological history rich in physiological problems. First and most important, it makes a prolonged and often exhausting journey without food other than that stored in its own tissues at the beginning. Second, it enters fresh water with the reproductive organs relatively immature and further development during the migratory journey is at the expense of other tissues. Finally, both males and females die after the spawning act. In a word, the king salmon makes a strenuous but foodless journey to its spawning beds, tarries there for the final maturing of its reproductive organs, spawns, and dies.

Miescher[1] and Paton[2] have studied the changes in the Atlantic salmon which returns to the sea for further feeding. Evermann[3] showed that the king salmon dies after spawning, and the present studies develop facts bearing on the causes of death. The

* Published by permission of the United States Commissioner of Fish and Fisheries.

[1] Miescher, F., Schweizerischer Fischerei-Ausstellung in Berlin, 1880, 154. Also reprinted in Die histochemischen und physiologischen Arbeiten von Friedrich Miescher, Leipsic, 1897, ii, 116.

[2] Paton, D. N., *Report of the Fishery Board of Scotland*, 1898, 143.

[3] Evermann, B. W., *Bull. U. S. Fish Commission*, 1895, xv, 253; 1896, xvi, 184.

changes in the blood,[4] the speed of migration,[5] the history of the fat deposits in the muscular tissues,[6] all aid in the interpretation of the chemical changes during the spawning migration.

Material.

The salmon used in this study were collected on the Columbia River from the mouth through five stations to the spawning grounds as follows: (1) Ilwaco is just at the mouth of the Columbia River. (2) Warrendale is 130 miles up the river and below the Cascades. (3) Seufert is on The Dalles of the Columbia and above the Cascades. These three stations are within the fields of the lower Columbia commercial fisheries. (4) Ontario is on the Snake River at the Oregon-Idaho line 700 miles above the sea and just below the spawning beds of the Payette River in the mountains of Idaho. (5) The Cazadero spawning beds are on the San Lorenzo River, a branch of the Willamette 30 miles above Portland and 130 miles from the sea. Spawning salmon taken here in September entered fresh water at Ilwaco with the spring migration between March and early May, and hence have been without food for 4 or 5 months.

There is a seasonal variation in both the food quality and the biological condition of the salmon entering the Columbia from the sea. The spring fish are rich in fats and of finer quality than summer and autumn salmon. Ilwaco salmon entering in August and September have more mature sex gonads and are inferior in flesh quality, chiefly in the store of fat. They spawn in the lower tributaries of the Columbia.

The muscle tissue is the principal storage tissue of materials both for energy production and for growth of sex gonads during migration, hence its importance for chemical study. Sets of muscle samples were analyzed from twenty-seven fishes of the forty-seven collected. The samples were collected in the order Seufert, Warrendale, Ilwaco, Cazadero, and Ontario. The standard fish from Ilwaco are for this reason probably below the seasonal average and by so much tend to minimize the changes presented.

[4] Greene, Chas. W., *Bull. U. S. Bureau of Fisheries*, 1904, xxiv, 429.
[5] Greene, Chas. W., *Bull. U. S. Bureau of Fisheries*, 1911, xxix, 131.
[6] Greene, Chas. W., *Bull. U. S. Bureau of Fisheries*, 1913, xxxiii, 69.

Selection of Analytical Samples.

Every effort was made to choose salmon typical for the station and season. Lengths near 80 and 100 cm. were chosen to diminish the errors of converting to the standard size. Both sexes were collected. The chemical samples were taken as soon as live fish from the fishing gear could be carried to the laboratory. Data as to weight, size, and form were taken by measurements and photographs.

The total mass of muscle decreases from 40 to 50 and more per cent, and there is a percentage change in the composition of the muscle protoplasm during the fast. To follow these changes it is obvious that the choice of chemical samples is critical and vital. We selected a median belt about 2 inches broad cut across the great lateral muscles of the left side at the level of the front border of the dorsal fin. The skin with subcutaneous fat was removed and the segment of muscle peeled off the skeleton. This mass was finely ground and mixed for uniformity. The method avoids the error of Paton's[2] arbitrary samples of the "thick" and the "thin" muscles, and gives a mixed sample of the high fat-containing superficial muscle, and the low fat-containing, deep lateral muscle.[7,8]

Duplicate series were preserved for water, for phosphorus and nitrogen, and for smaller (20 gm.) and larger (30 gm.) analytical samples. All the samples were weighed by difference in the permanent glass containers. The analytical samples were preserved in absolute alcohol in 70 and 90 cc. wide-mouth glass-stoppered jars. The attempt was to preserve in 80 per cent alcohol taking into account the diluting power of the water only. The finely divided sample was thoroughly stirred and the stopper sealed with hard paraffin at a temperature of the sample of approximately 50°C. This produced a partial air vacuum to prevent breaking the seal by air temperature change in shipping. It also favored alcohol coagulation and the arrest of autolytic processes. That the method successfully stopped the autolytic

[7] Greene, Chas. W., *Am. J. Anat.*, 1912, xiii, 175.

[8] Greene, Chas. W., and Greene, Carl H., *Bull. U. S. Bureau of Fisheries*, 1913, xxxiii, 21.

processes is proved by the analytical results, although the preserving concentration of alcohol is somewhat under the limit set by Wells and Caldwell.[9,10]

Methods.

Samples were analyzed by the methods of Koch[11] and of the Official Agricultural Chemists,[12] both modified in adaptation to the problems peculiar to the salmon muscular tissues. The essential point in the procedure is that the fresh tissue shall be coagulated and then thoroughly extracted with alternate alcohol and ether each proceeding as nearly as possible at the boiling points of the solvents. It is assumed that the difficultly soluble lipins will be freed by the hot alcohol treatment and then dissolved by the ether extraction which follows.

The samples were first filtered through Gooch crucibles. The filtrate with its excess of oil was set aside and the residue extracted in a modified Soxhlet[13] particularly adapted to procure extraction at the boiling point of the solvent, first in alcohol then in dry ether for 6 to 8 hour periods each for two successive extractions. The soluble fractions were added to the mother liquor and evaporated to dryness over a water bath. The dry residue was taken up in acidulated chloroform water which dissolves all but the lecithins and fats. The lecithin-fat emulsion was repeatedly washed with acidulated chloroform water, then oxidized, and a gravimetric phosphorus determination made as a

[9] Wells, H. G., and Caldwell, G. T., *J. Biol. Chem.*, 1914, xix, 57.

[10] Autolysis, of course, would reduce the amount of protein in the tissue and increase the extractives. These samples of the smaller size were analyzed in 1909, during the year and a half following the collection of the samples. The set of larger samples analyzed in 1916–17 by Carl H. Greene, see this *Journal*, page 457, were of the same collection. The close check of the total per cent of protein figured as fat-free tissue in the material used in the two papers, especially in view of the variations in analytical methods used, argues strongly for the accuracy of the method of preservation.

[11] Koch, W., *J. Am. Chem. Soc.*, 1909, xxxi, 1329. Koch, W., and Woods, H. S., *J. Biol. Chem.*, 1905–06, i, 203.

[12] Official and provisional methods of analysis, *Assn. of Official Agric. Chemists, U. S. Bureau of Chemistry, No. 107*, 1907.

[13] Greene, Chas. W., *J. Biol. Chem.*, 1909–10, vii, 503.

basis for computing the lecithins. The neutral fats were estimated by difference. The acid-water extract was evaporated in platinum over a water bath, dried at 105°C. to constant weight, and ashed in a muffle furnace. Phosphorus determinations made on all ash fractions are held for later report. The ash, the organic extractives, and the lecithins present in the alcohol-ether-soluble fraction were computed from these figures.

The alcohol-ether-insoluble residue was dried to constant weight at 105°C., repeatedly extracted in distilled water, the soluble fraction evaporated in platinum over the water bath, dried to constant weight at 105°C., and ashed in a muffle furnace as before. From these figures were obtained the insoluble coagulated protein residue, and the organic extractives and ash not removed during the alcohol-ether extractions. The two organic fractions and the two ashes were combined in the totals.

The water was obtained from separate fresh samples dried to constant weight while in the field. The neutral fat was computed by difference. On the whole it is believed that this result has proved acceptably accurate for the fats.

Laboratory equipment was taken in two different seasons to the collecting fields where analyses were made on fresh samples to test the adequacy of the sampling and preserving methods. It was on the basis of these early determinations that the final collecting and preserving technique was adopted.[14]

Of the twenty-seven fishes analyzed all were in duplicate except one, No. 1294. The grand averages are drawn from three fish from Ilwaco, four from Warrendale, six from Seufert, seven from Ontario, and seven from Cazadero. The range of variation among the individuals of a station is very little in such stable content as protein, etc., but, as one may expect, the variation is greater in the fat which is the primary stored food product.

[14] In 1904 such a laboratory was maintained in a corner of the P. J. McGowan and Sons cannery at Ilwaco, thanks to their scientific interest and courtesy in providing space, running water, and live steam. In 1906, through the courtesy of the Directors we enjoyed the better facilities of the Hopkins Seaside Laboratory of the Stanford University where sea run salmon were analyzed from Monterey Bay. Both stations yielded data sufficient to establish the success of the methods adopted for preserving material for analysis in the home laboratories.

Tables I, page 92, and II, page 115, Greene,[6] give additional data on the wide range of fat variation at Ilwaco and at the spawning grounds. The extremes are greater as the journey proceeds, due doubtless to the unequal supply of fats stored at the start. The same argument probably holds for the compound fats.

Method of Computing Data.

The analytical figures are presented in three different sets of computations. The first computation is based on the total wet sample, the second on the dry sample; *i.e.*, the water-free sample. These two are the computations usually followed by chemists. However, neither satisfies the demands of the biological problem we are attacking, since neither method shows the actual protoplasmic basis.

Certain tissues have long been known to store large quantities of inert carbohydrate and fat as food material. The liver is the classic example of a glycogen-storing organ. Claude Bernard three quarters of a century ago demonstrated a high per cent of stored glycogen in the liver and gave us our present conception of the glycogenic function of this organ. Fats have long been known as temporary constituents at least of the epithelial cells of the alimentary canal. Ever increasing evidence points to the constant presence of fats in other normal tissue cells such as the liver, the kidney, muscle fibers, etc., but it is only since 1900 that physiologists have spoken of a lipogenic function; *i.e.*, of the liver.[15]

In computing the variations in the chemical composition of any protoplasm of an organ, such as the liver, one should eliminate the inert factors of stored glycogen and stored fat if he would arrive at the true protoplasmic basis. In the salmon there is no stored glycogen in either the liver or the muscles, or at the most only a trace. Fat takes its place and the chief fat storage tissue is the muscle. We have previously shown that there is an enormous storage of fat not only *among* the muscle fibers but *within* certain types of muscle fibers.[6] This fat may amount to more than 30 per cent in the superficial muscle of salmon from Ilwaco. It seems no more a part of the active functioning proto-

[15] Loevenhart, A. S., *Am. J. Physiol.*, 1901–02, vi, 331.

plasm of the muscle of the salmon than is the fat in the adipose cells of the perimysium of the muscles of man or the domestic animals. The chemical computation which includes this store of inert fat is adequate for the determination of the food and fuel value of the muscle as such, but it is of no biological value in determining the degree of change in physiological characteristics of the tissue during the migration.

I have therefore computed the analyses on a third basis, that of the fat-free weight of salmon muscle. *The assumption is that when the stored fat of salmon muscle is eliminated from computation the tissue left represents the true protoplasm, the active living tissue.* This point is emphasized since the comparisons signifying the biological changes are based on the results obtained by the protoplasmic basis method of calculation.

Composition of Muscle Tissue from Different Stations.

Ilwaco Station at the Mouth of the Columbia River.

Salmon from the mouth of the Columbia River are the standard normal fish against which all other specimens are compared. These salmon are in the best of physiological condition and yield the choicest food product. If one were to compare the salmon with our domestic animals, for example, beef cattle, this stage from the Columbia tide-waters would represent the finished product in salmon flesh—the choicest in nutritive value, in delicate flavor, in all that contributes to a delicious food. Therefore, biologically and commercially this stage of the salmon life cycle represents the crest of its curve of physical development. Factors of chemical change are correlated as against this type.

In a previous study[6] attention was called to the types of variation in salmon condition that may occur even at the mouth of the Columbia. The biological basis of these variations has not yet been adequately explained. Nevertheless, the fact was recorded that salmon caught during the first 2 weeks in August actually varied in the fat content of the pink muscle between 2.7 and 20.2 per cent. The dark muscle fat in the same series varied between 14.3 and 30.8 per cent.

The salmon muscle of the present series contains an average fat content of the composite or mixed sample of 15.5 per cent with

TABLE I.

*Composition of the Muscle Tissue of the King Salmon, Oncorhynchus tschawytscha, during the Spawning Migration.**

Collecting stations.	Date.	Salmon No. and sex.	Water.		Solids.		Protein.			Phosphorized fats.			Neutral fat.		Organic extractives.			Ash.		
			Wet.	Fat-free.	Wet.	Fat-free.	Wet.	Dry.	Fat-free.	Wet.	Dry.	Fat-free.	Wet.	Dry.	Wet.	Dry.	Fat-free.	Wet.	Dry.	Fat-free.
	1908		*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Ilwaco, Wash.	Aug. 15	1276♂	62.5	74.5	37.46	25.30	16.92	45.16	20.16	0.76	2.56	1.14	16.1	42.9	2.62	6.99	3.12	0.89	2.36	1.05
Tide-water.	" 18	1279♀	63.8	74.6	36.23	24.88	17.25	47.60	20.18	1.03	2.84	1.21	14.5	40.1	2.35	6.55	2.75	1.06	2.92	1.24
	" 20	1281♀	63.2	75.2	36.80	24.78	16.73	45.46	19.88	1.00	2.71	1.18	15.8	43.0	2.48	6.74	2.95	0.73	2.07	0.91
Average			63.2	74.8	36.83	24.99	16.97	46.07	20.07	0.93	2.70	1.18	15.5	42.0	2.48	6.76	2.94	0.90	2.45	1.07
Warrendale, Ore.	Aug. 5	1259♂	60.3†	75.4	39.66	24.58	16.32	41.15	20.40	0.77	1.94	0.96	20.0	51.2	1.80	4.55	2.25	0.78	1.94	0.98
100 miles from	" 5	1261♀	63.4	74.3	36.57	25.59	17.11	46.80	20.04	0.60	1.62	0.70	15.1	39.9	3.16	8.65	3.71	0.94	2.56	1.10
the sea.		1262♂	62.2	75.8	37.76	24.21	16.15	42.79	19.67	0.75	1.99	0.92	17.9	47.3	2.15	5.71	2.62	0.82	2.17	1.00
	" 7	1263♀	64.7	74.4	35.28	25.58	17.96	50.90	20.65	0.57	1.62	0.66	13.0	36.9	2.84	9.04	3.26	1.03	2.93	1.19
Average			62.7	75.0	37.32	24.99	16.88	45.41	20.19	0.67	1.79	0.81	16.5	43.9	2.49	6.99	2.96	0.89	2.40	1.07
Seufert, Ore.	July 11	1040♂	64.5	75.2	35.46	24.79	17.39	49.05	20.27	0.95	2.67	1.10	14.2	40.0	2.02	5.71	2.36	0.91	2.56	1.06
210 miles from	" 12	1056♀	62.9	74.5	37.10	25.47	16.88	45.49	20.00	0.80	2.16	0.95	15.6	42.0	2.89	7.79	3.43	0.93	2.51	1.10
the sea.	" 13	1060♀	63.2	76.4	36.83	23.58	16.27	44.19	19.65	—	—	1.06	18.2	49.5	1.57	4.26	1.90	0.77	2.09	0.93
	" 15	1074♂	60.3	74.9	39.67	25.12	16.42	41.29	20.38	0.87	2.19	1.09	19.4	48.9	2.04	5.13	2.53	0.89	2.23	1.10
	" 23	1179♂	63.4	74.0	36.56	26.01	17.60	48.13	20.52	0.78	2.14	0.91	14.2	39.0	2.92	7.98	3.40	1.01	2.76	1.18
	" 31	1180♀	65.8	74.2	34.21	25.83	17.49	51.13	19.72	0.75	2.19	0.85	11.3	33.0	3.47	10.14	3.91	1.20	3.51	1.35
Average			63.4	74.9	36.64	25.15	17.01	46.55	20.09	0.83	2.26	0.96	15.5	43.2	2.49	6.84	2.92	0.95	2.61	1.12

Ontario, Ore. 700 miles from the sea.	Sept. 6	1312 ♀	70.4	76.2	29.62	23.81	17.36	58.61	18.79	0.63	2.11	0.67	7.3	24.7	3.14	10.61	3.40	1.16	3.90	1.25
	" 7	1314 ♂	68.8	77.0	31.22	23.03	16.19	51.85	18.11	0.52	1.66	0.73	10.8	24.5	2.69	8.63	3.01	1.05	3.35	1.17
	" 7	1315 ♀	68.2	77.2	31.82	22.81	15.87	49.88	17.96	0.14	0.45	0.16	11.7	36.6	3.21	10.10	3.62	0.98	3.08	1.10
	" 8	1316 ♂	67.4	76.5	32.60	23.46	16.50	50.54	18.71	0.20	0.60	0.22	11.9	36.6	3.00	9.21	3.41	1.07	3.28	1.21
	" 7	1317 ♂	71.2	76.7	28.82	23.24	17.33	60.14	18.69	0.28	0.98	0.30	7.3	25.2	2.96	10.26	3.18	0.99	3.43	1.07
	" 9	1321 ♀	69.0	77.8	30.98	22.20	15.90	51.31	17.91	0.37	1.20	0.42	11.3	36.4	2.52	8.15	2.84	0.90	2.92	1.00
	" 11	1326 ♀	67.9	77.8	32.11	22.19	15.05	46.87	17.25	0.19	0.60	0.22	12.7	39.7	3.07	9.56	3.52	0.87	2.70	0.99
Average			69.0	77.0	31.02	22.96	16.31	52.74	18.20	0.33	1.08	0.39	10.4	33.4	2.94	9.50	3.29	1.00	3.24	1.11
Cazadero, Ore. The spawning grounds.	Aug. 25	1294 ♀	80.2	80.8	19.77	19.20	14.48	73.17	14.58	0.45	2.26	0.45	0.7	3.5	3.08	15.57	3.10	1.06	5.37	1.07
	" 25	1295 ♂	78.1	80.5	21.89	19.51	14.41	65.83	14.85	0.44	2.01	0.45	2.9	13.5	3.17	14.50	3.27	0.91	4.17	0.94
	" 26	1296 ♀	78.8	81.8	21.21	18.18	13.37	63.05	13.88	0.18	0.84	0.18	3.7	17.4	3.00	14.17	3.12	0.96	4.51	0.99
	" 26	1297 ♀	81.4	83.0	19.56	17.06	12.82	69.09	13.05	0.29	1.56	0.29	1.8	10.2	2.71	14.63	2.76	0.87	4.78	0.90
	" 27	1298 ♂	81.0	82.2	18.97	17.79	13.62	71.79	13.82	0.45	2.35	0.45	1.4	7.6	2.69	14.17	2.73	0.98	5.19	1.00
	" 27	1299 ♀	79.3	81.8	20.68	18.15	12.85	62.16	13.26	0.62	3.02	0.64	3.1	14.9	3.22	15.58	3.32	0.90	4.34	0.93
	" 28	1300 ♂	78.8	80.6	21.19	19.44	14.41	67.99	14.73	0.60	2.86	0.62	2.2	10.3	3.09	14.57	3.16	0.91	4.29	0.93
Average			79.7	81.5	20.32	18.48	13.71	67.58	14.02	0.43	2.13	0.44	2.2	11.1	2.99	14.74	3.07	0.94	4.66	0.97

* The series consists of three specimens from Ilwaco at the mouth of the Columbia River, four from Warrendale below the Cascades, six from Seufert fishery at The Dalles, seven from Ontario on the Snake River, and seven from the spawning grounds at Cazadero on the San Lorenzo River. The spawning salmon have been in fresh water without eating for a time estimated from 4 to 5 months.

The figures are averages of duplicate samples, with the single exception of No. 1294. The computations are made on three bases,—the fresh wet sample as taken, the dry sample, and the fat-free sample.

† The water determination of this sample was substituted from the analysis of Carl H. Greene.

extremes of approximately ±0.5 as variations. This is the best measure of the success in choosing typical and average salmon for analyses.

The water determinations made on separate small samples, selected at the time of collecting analytical muscle samples, average 63 per cent of the total sample for these Ilwaco specimens. When the water is computed on the protoplasmic or fat-free basis the average is 74.8 per cent and the extremes are 74.5 and 75.2 per cent in the three fishes analyzed. The protein content is even more strikingly constant. On the protoplasmic basis it averages 20.07 per cent and the variation is less than 0.2 per cent. The organic extractives average 2.94 per cent, the lecithin 1.18 per cent, and the inorganic ash 1.07 per cent (Table I).

The greatest variation in tissue composition at this station is found in the fats. But the three fishes analyzed proved very constant in this particular variant. Confidence is felt in the samples of salmon presented as average types. The four fish from Warrendale and the six from Seufert also check closely as the results show.

Salmon from Warrendale.

Four salmon were analyzed from this station all collected August 5th and 7th. Warrendale is below the Cascades of the Columbia and salmon have not yet fought these turbulent river currents in their migration. Although these fish vary in size and sex, the composition of their tissues varies little from the standard from Ilwaco station. Apparently the migration has not seriously affected the proportionate distribution of chemical groups in the tissues. The collections being made a little earlier in the season than the Ilwaco standard introduces a seasonal factor in favor of this station. The average results are given in Table II.

Salmon from Seufert.

Six salmon from the station at Seufert were analyzed. Seufert is approximately 80 miles up river from the Warrendale station. To make this run, the fish must pass the long and turbulent rapids of the Cascades as well as the rapids of The

Dalles. In fact the entire distance is through the Columbia River canyon in the Cascade Mountains and the currents are swift and strong. It is obvious that the salmon must swim for many miles more than the actual distance between the two stations. The Seufert collection was made between July 11th and 31st. This is an average of 4 weeks earlier in the season than the Ilwaco collection used as the standard. When the seasonal variation is ultimately determined a correction factor can be applied to equalize this unknown deviation. At present all one can do is to assume on the basis of the known general variations of quality that the observed percentages are comparatively high at Seufert.

By reference to Table I it is noted that the Seufert fishes represent wider extremes as to fat content. They are equally distributed as to sex. The muscle water on the protoplasmic basis is 74.9 per cent. This is an increase so slight over that observed at Ilwaco as to be quite within the limits of error in observation. The proteins agree exactly with the Ilwaco percentages. The fats average 15.5 per cent but the individual variations are from 11.3 to 19.4 per cent, rather wide extremes.

Salmon from Ontario Station on the Snake River.

The Ontario collection of seven analyzed salmon was made during the 2nd week in September. The journey of 700 miles made by these salmon includes the Canyon of the lower Snake River in addition to that of the Columbia. The per cent of the water of the total tissue amounts to only 69. But computed on a protoplasmic basis the muscle water averages 77 per cent. There is a variation among individuals of less than 0.8 per cent. The muscle proteins average 18.2 per cent with a variation between 17.25 and 18.79 per cent. The neutral fats average 10.4 per cent but the extremes observed show a wider range of variation in fat content; *i.e.*, from 7.3 to 12.7 per cent. This variation bears no obvious relation to the variations either in water or protein content considered from the protoplasmic basis point of view. The phosphorized fats average 0.39 per cent. The extremes are great, 0.16 to 0.73 per cent, but the absolute quantity is small. In such small quantities the gravimetric phosphorus method is not sufficiently accurate. The ash averages 1.11 per cent and is the same as that found at Seufert.

The organic extractives average 3.29 per cent for the station, a fact that may be better understood when interpreted in light of the fact that at this station fish have only recently completed the run through the lower Snake River Canyon. The extractives are over 3 per cent in all individuals save one. They seem constantly higher than at the lower river stations. The stress of migration is at its height here and muscle protein disintegrative processes are maximal. It is assumed that the kidneys are actively eliminating these extractives throughout the journey. Nevertheless, there is enough lag for the concentration to increase in the muscle tissues (see Table II). This increase amounts to over 10 per cent although the water content increases only a trifle over 2 per cent. The proteins from which the extractives are derived have decreased from 20 to 18.2 per cent, a fact that supports the inference of augmented catabolism at this station.

Cazadero Station.

The collection at Cazadero represents the final and most interesting link in the chain of stations. Salmon are spawning here and dying. Fish to all external appearances that are well and vigorous may be found dying 24 and 48 hours later. Our collection of the seven analyzed fish was made August 25th to 28th. These salmon entered the Columbia with the spring run, presumably in April or earlier. They had been in fresh water and without food for 4 months or more.

The muscle fats average 2.2 per cent. The extremes are wide, from 0.7 to 3.7 per cent. The two fish yielding the extremes are females, hence the variation cannot be charged to the fact of excessive use of fats in the growth and maturing of the large mass of the ovaries. The water of the fat-free muscle averages 81.5 per cent. The individual variation is from 80.5 to 83.0 per cent. Three of the four females analyzed have a muscle water content above the average for the station. This suggests that the sex factors are contributory to the tissue changes expressed by the increasing water content.

The muscle proteins are very low, an average of 14.01 per cent computed on the protoplasmic basis. The figure is relatively constant for the station; *i.e.*, the extremes are only from 13.05 to

14.85 per cent. The females average 0.3 per cent of protein lower than the average for the station. This difference strongly suggests that the growth of ovaries is a heavy drain on the muscle tissue. The lecithins are 0.44 per cent, the organic extractives 3.07 per cent, and the ash 0.97 per cent for averages. The high content of extractives is surprising in view of the great decrease in protein from which the organic extractives arise. The degree of saturation in the muscle water drops back to 3.8 per cent, which agrees with the saturation of 3.9 for the stations on the lower river. This supports the contention of Carl H. Greene that the muscle water may vary but that the extractives maintain a comparatively constant degree of saturation. Since the water of the muscle is in round numbers 81.5 per cent, the organic extractives amount to 3.85 per cent of the muscle water. The ash content is 0.97 per cent.

Biological Significance of the Results.

Variation in the Amount of Neutral Fat.

The immediate source of kinetic energy expended by the salmon during the spawning migration is without question to be found in the neutral fats. There is no food intake to supply energy-yielding substances. Little or no glycogen is present in the tissues. But there is always an abundance of fat. It has been shown by histological studies that the fats are present not only between the fibers but are distributed in most intimate relation to the fibrillar structure of the individual muscle fibers. Fat is maintained in this relation during the entire migration even though the intermuscular fat decreases to a low ebb.[6] Chemical analyses show that the grand average percentages of fat for the samples analyzed at the different stations are as follows: Ilwaco 15.5, Warrendale 16.5, Seufert 15.5, Ontario 10.4, and Cazadero 2.2 per cent (Table II). There is, as one would expect, much variation among individual fishes at each station, a variation that is most extreme in the Ontario and Cazadero stations. At Ilwaco the variations amount to only a little over 1.5 per cent among the fishes analyzed. But this in terms of the average fat is a variation of some 10 per cent of the total. There is enough of a handicap to the salmon of low fat-supply to account for the

much greater variations noted among individuals at the upper stations. A salmon beginning the migration with a low store of fat will the more quickly be exhausted by the process.

The total average percentage decrease of fat is from 15.5 per cent in terms of wet tissue at Ilwaco to 2.2 per cent at the spawning grounds. This is an apparent loss of 85.5 per cent of the stored fat. However, the total consumption of fat is much greater, about double. This is accomplished by the 40 to 50 per cent total reduction in the mass of the tissue as well as the change in percentage composition of that remaining.

TABLE II.

Summary of Changes During the Migration.

Station.	Distance from sea.	Protein.	Neutral fat.	Lecithins.	Organic extractives.	Ash.	Water.
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Ilwaco	Tide-water.	20.07	15.5	1.18	2.94	1.07	74.8
Warrendale	130 miles.	20.19	16.5	0.81	2.96	1.07	75.0
Seufert	210 "	20.09	15.5	0.96	2.92	1.12	74.9
Ontario	700 "	18.20	10.4	0.33	3.29	1.11	77.0
Cazadero	Spawning grounds.	14.02	2.2	0.44	3.07	0.97	81.5

These figures strongly support the contention that the primary source of the energy expended by the salmon during the abstinence from foods is found in the stored neutral fats.

Variations in Lecithins.

The samples analyzed yielded rather too small fractions of lecithins for best accuracy in their determination by the gravimetric phosphorus method. Nevertheless, very definite variations are revealed by comparing the averages for the stations. The average for Ilwaco is highest, 1.18 per cent of the tissue computed on the protoplasmic basis. The next two stations within the fishing limits of the Lower Columbia, Warrendale and Seufert, average 0.81 and 0.96 per cent respectively. This seems to show a decided early decrease in phosphorized fat during the migration. If these three stations are averaged the figure for the thirteen fishes is 0.98 per cent. The salmon of the Ontario

station yielded only 0.33 per cent of muscle lecithins, and the spawning salmon at Cazadero 0.44 per cent.

A convenient standard tissue determination is Bloor's[16] analysis of human blood. Red blood corpuscles yield 0.40 per cent of lecithin. The normal salmon at the mouth of the Columbia with 1.18 per cent of muscle lecithin contain three times that of human corpuscles. However, even at Warrendale, after a journey of 130 miles the lecithins have dropped off 30 per cent of the total. At Seufert the loss was 20 per cent, a higher lecithin content than found at Warrendale. The extremes observed at these two stations, though great, yielded no individual with as high a lecithin content as observed in fishes at Ilwaco.

The lecithin average for the Ontario and Cazadero salmon agrees with normal human blood corpuscle lecithin; *i.e.*, 0.33 and 0.44 per cent in comparison with Bloor's 0.40 per cent for red corpuscles. The amount indicates a loss of from 60 to 70 per cent of the lecithins present in the Ilwaco normal. In round numbers the muscle has used two-thirds its phosphorized fat. Considering the mass loss of muscle the total lecithin of the muscle still retained is only one-fifth its original total. This showing argues in favor of the active and vital part lecithin is generally understood to play in metabolism. It does not strongly support the view that the neutral fats pass through a phosphorized stage in muscle tissue metabolism. In the growth of the developing ovaries there is a somewhat higher lecithin content laid down; *i.e.*, as much as 4 per cent in the ovaries in comparison with the 1.18 per cent in muscle. It is generally assumed but not proved that the ovarian lecithin is synthesized lecithin. The above observations suggest strongly that the ovarian lecithin may be only transported or mobilized lecithin in the case of the salmon. If so, the muscle is the storage tissue from which the ovarian lecithins come.

The Muscle Proteins.

Our method of analysis heat-coagulates the proteins in the presence of alcohol, hence all the muscle proteins appear in one fraction. The protein of the normal prime salmon tissue figured

[16] Bloor, W. R., *J. Biol. Chem.*, 1916, xxv, 586.

on the protoplasmic basis varies only slightly from 20 per cent; *i.e.*, 20.07 at Ilwaco, 20.19 at Warrendale, and 20.09 at Seufert. These averages for the three stations on the lower river reveal an unexpected uniformity of composition. The variations among the thirteen salmon analyzed are within the limits of experimental error.

The average protein of the seven analyzed salmon from Ontario is 18.20 per cent, with extremes from 17.25 to 18.79 per cent. The variations are greater at this station. The decrease in average amount of protein from 20.07 per cent at Ilwaco to 18.20 at Ontario is of special significance. It is true that the water content increases, but the two factors are not parallel. Obviously the long fast and the profound output of energy is wearing on the muscle as an energy-producing machine. The full significance of this change is more fully revealed by the condition observed at the spawning station.

All seven salmon analyzed from the spawning grounds at Cazadero show a great decrease in the per cent of protein. The average content of the muscle for the station is 14.02 per cent, a loss per unit weight of tissue of 6.05 per cent. This is a loss of 30 per cent of the total protein present in the muscle at the beginning of the migration. In the extreme case of No. 1297, the loss is 35 per cent of the standard protein per unit of tissue. The percentage variation within the group is not so great as one would anticipate. The highest is No. 1295 with a percentage of 14.85. The lowest is No. 1297 with 13.05 per cent of muscle protein. The first is the smallest fish in the group, while the second is intermediate in length. The largest fish analyzed is the female No. 1296 weighing 12.91 kilos. The protein of the muscle is 13.88 per cent, a shade under the station average. It is evident that the falling off of the percentage of protein of these salmon affects all fishes in similar ratio and independent of size, length, or other individual physical factors. No. 1295 is a male. All the others used in the above comparisons with No. 1295 are females. The factor of sex is the best clue to the maintenance of the protein percentage in the male at the highest level observed for the station. In the females a much larger amount of protein is built into the developing ovary than is built into the testes of the male.

The comparative constancy of percentile composition in protein checks well with the evidence derived from histological studies and indicates a maintenance of normal structure in the presence of prolonged abstinence from food.

The facts just advanced justify calling attention to the real significance of the changing protein concentration in the muscle protoplasm. Before the American Society of Biological Chemists at the Minneapolis meeting in 1917[17] it was argued that *these facts indicate a storage of reserve protein in the salmon muscle.* The argument is strengthened by two supplementary facts. The first of these is that the normal histological structure of the tissue is maintained to the very last days of the salmon life. I have never seen a clear case of degeneration of the type of the Zenker's degeneration of human pathology in the great lateral muscle either in the midlateral area or in the more exhausted muscle of the caudal peduncle, though I have noted it in the masseter muscle. The midlateral and the caudal peduncle are the regions from which series of samples were taken in the comparative studies by histological methods of the fat distribution. The striated and fibrillar structure of the muscle was found to be maintained in striking clearness.[6] The second point is the fact that the great lateral muscles are physiologically active and function normally until near death. A spawning salmon is still capable of dealing a most vigorous swimming stroke and must make similar movements in expelling the eggs. The activity and the structure both signify normal muscle, notwithstanding the decrease of protein by 30 per cent or more. The corollary is that the normal muscle contains an amount of protein in excess of its functional requirements. This protein reserve cannot be demonstrated by such simple histological methods as we possess for identifying reserve glycogen, or reserve fat.

The protein storage hypothesis greatly aids the interpretation of many known phenomena. For example, the usual conception of the protein relations in body tissues assumes that each protein molecule is an integral part of the bioplasmic unit. To free protein, either directly or indirectly, through cleavage into the constituent amino-acids, is by the current view to destroy so many

[17] Greene, Chas. W., *J. Biol. Chem.*, 1918, xxxiii, p. xiii.

units of living tissue. Conversely, the utilization of amino-acids by the animal can by this same view take place only by the construction of new tissue. Van Slyke and Meyer[18] have established two very fundamental facts. The first is that muscles absorb amino-acids during the time of their excess in the blood up to a quite definite saturation point. Also that this point is independent of, or at least greatly in excess of, the saturation point in the blood at equilibrium. The second fact is that in fasting the muscles may actually increase in amino-acid content. Their deduction from these facts is stated as follows: "The amino-acids appear, therefore, to be intermediate steps, not only in the synthesis, but in the breaking down of body proteins."

If one admits that proteins may be attached to the cell protoplasm in some such way as are carbohydrates and fats, the hydrolysis of which presents no very drastic event in so far as the unit protoplasmic structure is concerned, then the observations of Van Slyke and Meyer are easier of interpretation and more significant. The real significance of the two essential observations of Van Slyke and Meyer is furnished by the determinations of Carl H. Greene[19] in the paper that follows. Greene demonstrates that the *muscle fluids* of the migrating salmon *remain saturated with amino-acids* to a constant level as the migration proceeds. The degree of saturation is independent of the variation of the amount of protein in the muscle but coincides with the increasing muscle water. We find in the present work that the organic extractives are augmented at Ontario station but constant at all the others (p. 446). This fact in conjunction with our observed decrease in the total amount of proteins greatly strengthens the deduction that there is a definite protein storage.

The argument that water is absorbed by the muscle as fat and protein are lost, and in direct proportion, and thus accounts for the percentage decrease of protein per unit of mass, is untenable. One must assume that the amount of water is determined either by osmotic pressures, or is held in colloidal solution. The osmotic factor is assumed to be dependent primarily on the amount of inorganic salts. The tabulated figures show relative con-

[18] Van Slyke, D. D., and Meyer, G. M., *J. Biol. Chem.*, 1912, xii, 399; 1913–14, xvi, 232.

[19] Greene, Carl H., *J. Biol. Chem.*, 1919, xxxix. 457.

stancy in the group, a decrease of ash occurring only at the spawning station and at the end of the life cycle. Colloidal water in the presence of a constant saturation of inorganic constituents should vary with the amount of colloid. As a matter of fact, the two concentrations vary inversely. With the ash content relatively constant, the water absorbed by the muscle colloids should vary in a direct percentile ratio to the amount of protein present. Such is not the case.

The Organic Extractives.

The organic extractives represent the waste products of protein metabolism. This fraction in the salmon muscle gives a direct measure of the disintegration of tissue proteins. Since excretion of nitrogenous wastes continues during the migration, it follows that any sudden or acute variation of the amount of these substances will ultimately be equalized to a certain degree by the processes of excretion. We are inclined to believe that the individual variations observed within a particular station are explained to some extent by this view.

The amounts of organic extractives averaged for the stations are presented in Table I. On the protoplasmic basis the figures are 2.94, 2.96, and 2.92 per cent for the three stations along the commercial fishing territory. At Ontario the concentration is higher; namely, 3.29 per cent. At Cazadero it is 3.07 per cent. The uniformity on the lower river is striking and the relation is the same whether the comparisons are made on the basis of the total protoplasm, or of the protein content, or of the tissue water.

The 3.29 per cent of the organic extractives at Ontario represents a definite augmentation at this stage. In fact only one of the seven fishes, No. 1321, fell below 3 per cent. However, the increase amounts only to 12 per cent of the extractives at Ilwaco. But when computed in per cent of the protein of the tissue it is a 23 per cent increase in the ratio of extractives to protein. This is very significant in view of the fact of the source of the extractives from a diminishing protein supply.

The Cazadero average of 3.07 per cent is still high as compared with the 2.94 per cent average at Ilwaco. This is not a very significant change considered as per cent of protoplasm. But

computed as an extractive-protein ratio the result is a surprise. The organic extractives amount to 21.9 per cent of the protein found at Cazadero. This ratio between organic extractives and protein is more constant for the station than the extractive-protoplasmic ratio, since salmon at this station with low extractives are as a rule also low in protein, and *vice versa*. The extractive-protein ratio shows an increase in the extractives of 50 per cent over the normal, and 22 per cent over the Ontario ratio.

In his study of the distribution of nitrogenous extractives in the salmon muscle supplied from this collection, Carl H. Greene[19] offers the significant explanation. He finds that the amino-acids remain in constant concentration in the muscle water after the catabolic mechanisms are once well established on the migration basis. My observation on the total organic extractives in the main support this view. However, my figures show an obvious increased degree of concentration of the organic extractives in the muscle waters at Ontario, the ratio of 39 to 43, but diminution to the original level at the spawning grounds (Table III). The Ontario ratio may possibly indicate an upset in the production-excretion balance due to the assumed increased metabolism.

TABLE III.

Organic Extractives of Salmon Muscle.

Station.	Protoplasmic basis.	Dry basis.	Wet sample basis.	Protein.	Muscle water.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
Ilwaco	2.94	6.76	2.84	14.6	3.9
Warrendale	2.99	6.89	2.49	14.2	3.9
Seufert	2.92	6.84	2.49	14.5	3.9
Ontario	3.29	9.50	2.94	18.0	4.3
Cazadero	3.07	14.74	2.99	21.9	3.8

The showing as to the organic extractives still further emphasizes the inference already drawn from the comparisons of proteins and the fats; namely, that salmon are in a stage of accelerated metabolism at the Ontario station. The fish are in a biologically aggressive stage of migration. At the spawning grounds energy metabolism has diminished, the tissues are less active, and there is a corresponding slight falling off of the saturation of the muscle

water with organic extractives. This decrease in saturation is insignificant but perhaps explained by the fact that the available proteins are reduced by 30 per cent of the initial normal at Ilwaco. Reasoning by analogy with the facts it would seem that the stored protein excess is approaching depletion.

The Inorganic Ash.

The inorganic constituents of salmon muscle remain remarkably constant until the very last or spawning station. The computations for the five stations figured on the protoplasmic basis average 1.07, 1.07, 1.12, 1.11, and 0.97 per cent respectively. The slight decrease in ash at Cazadero is just about compensated for by the increase of tissue water.

SUMMARY.

In this study of the migrating king salmon, *Oncorhynchus tschawytscha*, twenty-seven salmon have been quantitatively analyzed for the chief group constituents of the muscle tissue. A large amount of new data is presented which not only measures the food value of the fish but which throws light on the nature of the protoplasmic changes during the fast of the long migration journey. The points to emphasize in summary are:

1. Computed on a fat-free tissue basis the muscle water remains constant through the lower river stations of Ilwaco, Warrendale, and Seufert, 74.8, 75.0, and 74.9 per cent, increases to 77.0 per cent at Ontario, and to 81.5 per cent at the spawning grounds. This is an augmentation of 6.6 per cent.

2. The inorganic ash of salmon muscle remains constant until the spawning station is reached when it diminishes slightly with the increasing water content of the tissue.

3. Neutral fats supply the chief energy for the kinetic metabolic processes during the migration. An average muscle fat content of over 15 per cent at the beginning of the migration is reduced to 2.24 per cent at its close, a total loss of 85 per cent of the fats.

4. The phosphorized fats or lecithins which are small in normal amount in muscle are nevertheless definitely consumed during the

journey. The gross change is from 1.18 per cent normal to 0.44 per cent at the spawning grounds.

5. The organic extractives are augmented in absolute amount in the late stages of the migration. The percentage ratio of organic extractives to protein is strikingly increased in the late stages, the ratio increase amounting to 50 per cent at the spawning grounds as compared with the normal. In relation to the muscle water the total organic extractives are relatively constant, obeying the law set forth by Carl H. Greene as to the constant saturation level of the muscle water by amino-acids.

6. The average protein content of the normal salmon muscle is 20 per cent when computed on a fat-free or protoplasmic basis. The proteins of muscle at the end of the migration average 14 per cent. Since the muscle remains physiologically active and vigorous the conclusion is drawn that *the 30 per cent of muscle proteins lost from the normal as shown by the composition of the spawning muscle is stored protein which the salmon can use in the emergency of migration without jeopardizing the protoplasmic integrity of the muscle.*

CHANGES IN NITROGENOUS EXTRACTIVES IN THE MUSCULAR TISSUE OF THE KING SALMON DURING THE FAST OF SPAWNING MIGRATION.*

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In the preceding paper by Charles W. Greene the general changes in the composition of the muscles of the salmon during the fast of the spawning migration are presented. The question at once arises as to the transformations occurring in this catabolism of the muscle protoplasm.

Since the time of Liebig a bewildering multitude of nitrogenous extractives has been isolated from muscular tissue. The physiological study of the greater number of these compounds, however, has always been hampered by the lack of adequate quantitative methods. Thanks to Folin and to Van Slyke, rapid and accurate methods are now available, especially for the determination of creatine and the amino-acids. These methods have been applied to the study of the changes in mammalian muscle during work and fasting. The enormous tax upon the muscle of the salmon during the spawning migration, however, greatly exceeds any that is usually met with in physiological experimentation, even of the most rigorous character. Consequently this material is most favorable as a basis for the study of the changes in the muscle extractives.

* The experimental data in this paper are taken from the dissertation presented by the author for the degree of Doctor of Philosophy, Yale University, 1917. A brief report was made before the American Society of Biological Chemists, December, 1917, see *J. Biol. Chem.*, 1918, xxxiii, p. xii.

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Recent Observations on Nitrogenous Extractives in Muscular Tissue.

Wilson (1914) reviewed the general literature on the occurrence of the different extractives that have been isolated from muscle. A somewhat similar bibliography is given by von Fürth (1909).

Extractive Nitrogen.

The total non-protein extractive nitrogen in mammalian muscle as determined by von Fürth and Schwarz (1911), Folin and Denis (1912), Buglia and Costantino (1912, *a*, *b*), and Cathcart (1916), varies between 250 and 390 mg. per 100 gm. of muscle. In the lower vertebrates Buglia and Costantino (1912, *c*) found values of 470 to 570 mg. per 100 gm., whereas in the invertebrates are found the largest amounts of all, 440 to 1,310 mg. per 100 gm. (Buglia, Wilson). In the elasmobranchs Buglia found the unusually high amount of 1.5 per cent of extractive nitrogen in the fresh tissue. This, however, is associated with the presence of large amounts of urea in the tissues.

Ammonia Nitrogen.

Buglia and Costantino (1912, *b*) demonstrated that the amino nitrogen in the muscles of mammalian embryos increased from 40 mg. per 100 gm. in a 2 months calf to 58 mg. in a full term fetus. They furthermore found the amino nitrogen in the muscles of the adult to vary between 60 and 90 mg. per 100 gm. Delaunay (1910) estimated from 168 to 252 mg. of amino nitrogen in dog muscle, but admits that his method was designed to give relative and not absolute values. These figures are undoubtedly high, as Van Slyke and Meyer (1913–14, *a*) proved that dog muscle becomes saturated when it contains approximately 80 mg. per 100 gm.

In common with their content of extractives, the muscles of invertebrates show a much higher content of amino nitrogen than do those of mammals. In *Sipunculus*, Buglia found 589 mg. per 100 gm. of fresh muscle. This is the highest reported figure for the amino nitrogen in muscle.

Van Slyke and Meyer (1912, 1913–14, *a*, *b*) demonstrated that an equilibrium existed between the amino-acids of the tissues and blood. On the injection of digestion mixtures into the blood stream it was possible to raise the amino nitrogen content of the body tissues. The muscles, however, reached a definite "saturation point" above which the amino nitrogen content could not be raised. The liver, on the other hand, very rapidly metabolized the absorbed amino-acids and would take up much greater amounts than the muscles. Cathcart (1916) also found that the composition of the muscle was less affected than was that of the liver.

In studies on the effect of different experimental procedures upon the amino-acid content of striated muscles, von Fürth and Schwarz (1911) were unable to affect the amino nitrogen of dog muscle by tetanic stimulation. Van Slyke and Meyer (1913–14, *c*) found no difference in the amino

nitrogen content of the muscles of dogs on a high or low protein diet. Wishart (1915) likewise found no increase in the residual nitrogen of dog muscle after a heavy meat feeding though there was a slight increase in the urea nitrogen. Mitchell (1918) obtained somewhat similar results in a study of the whole carcass of rats under different dietary conditions. He believes, however, that in young animals a demonstrable increase in the amino nitrogen occurs about 5 hours after feeding. Buglia and Costantino (1913) and Van Slyke and Meyer (1913–14, *c*) demonstrated that in fasting dogs the muscles contain more amino nitrogen than normally, approaching the saturation limit of Van Slyke. Reference may also be made to Van Slyke's (1915–16) recent summary of the physiology of the amino-acids.

Creatine Nitrogen.

Myers and Fine (1913, *a*) have recently reviewed the chemistry of creatine in the muscle in great detail and reference for the extensive literature on the subject may be made to their papers.

Though creatine is present only in vertebrates and the amounts are distinctive for the species, there is no marked phylogenetic relationship in the amounts present in the muscles. Rabbit muscle contains more creatine (0.52 per cent) than that of man (0.39 per cent). Grindley and Woods (1906–07) and Mellanby (1907–08) found that the muscles of the lower vertebrates, frogs and fishes, contain relatively small amounts of creatine, 0.28 to 0.35 per cent. In a number of teleosts Okuda (1912) determined creatine in quantities of from 0.42 to 0.75 per cent. In salmon muscle he found 0.55 per cent. These results of Okuda are probably high; certainly they are not in agreement with the analyses of Mellanby, or the experiments of Suzuki and Joshimura (1909) on the direct isolation of creatine from fish muscle.

Since the time of Liebig much attention has been paid to the variations in the creatine content of muscle under different experimental conditions. The earlier studies were made by the Salkowski-Neubauer method and are consequently not reliable. Since the introduction of the Folin method for the determination of creatinine several papers have appeared upon the changes in the muscle creatine. Thompson (1917–19) has recently summarized the present views as to the effect of work on the amount of creatine in the muscle. His own experiments made on decerebrated cats demonstrated that there was no change in muscles with an intact circulation after intermittent stimulation for over 2 hours. On the other hand, when the circulation was interfered with by arterial ligation the stimulation resulted in a loss of over 6 per cent of the muscle creatine. Similar or even greater losses occurred in the total nitrogen and solids of the affected muscle. Accordingly Thompson believes that with an intact blood supply there is a complete restitution of the substances used up by the muscular contraction, but that if the blood supply is restricted there is a reduction in the energy-yielding material of the muscle. This loss involves not only the non-nitrogenous substances but the total nitrogen and creatine of the

muscle as well. In contrast to these results, Scott and Spohn (1916–17) report that fatigue of the isolated muscle has no effect upon the total creatinine, but that fatigue of the living cat results in a loss of from 18 to 30 per cent of the creatine in the muscles. The views of Pekelharing (1911) as to the dependence of the creatine content of a muscle upon the condition of tonus rather than upon the direct muscular activity are well known.

There has been even greater confusion concerning the behavior of the muscle creatine during starvation than during muscular work. Myers and Fine (1913, *b*) put forward the following view.[1]

"The experiments . . . . indicate that the creatine concentration of the muscle in the rabbit is first increased during starvation, then subsequently decreased. The increase is apparently due to the removal of the non-creatine portion, *e.g.*, glycogen, fat, etc., of muscle more rapidly than that containing creatine. The elimination of creatine in the urine increases with the length of fast and in the days preceding death relatively large amounts are eliminated. This results in a depletion of the creatine supply of the body and in a decreased content of muscle creatine. . . . . The loss of creatine in the urine is still, however, the most important factor in causing a depletion in the creatine content of the muscle."

Benedict and Osterberg (1914) working on fasting dogs secured results agreeing with those of Myers and Fine.

Howe and his coworkers (1911, 1912) emphasized most strongly the possibility of washing out the creatine from the muscle without affecting the other constituents but their conclusions have been strongly questioned by Mendel and Rose (1911–12) and by Cathcart and Orr (1914). The belief of Urano (1907) and of Folin and Denis (1914) that creatine is an integral part of the muscle protoplasm is also opposed to this view of Howe and Hawk.

Materials Investigated.

The samples of salmon muscle used were duplicates kindly supplied for this study of nitrogenous extractive distribution by Professor Charles W. Greene from his research collection taken on the Columbia River during the summer of 1918 and reported in this *Journal*, page 435. The analytical samples were selected, weighed, and preserved in alcohol in glass-stoppered bottles and sealed by paraffin as described in his paper. The samples are a composite from the middle section of the great lateral muscle described by Greene (1912), and Greene and Greene (1913).

[1] Myers, V. C., and Fine, M. S., *J. Biol. Chem.*, 1913, xv, 301.

The chemical examination was completed in the Sheffield Laboratory of the Yale University, during the winter of 1916–17.[2]

The majority of the samples used were of the larger series and varied between 28 and 37 gm. The total volume of the alcohol and sample was from 90 to 100 cc., so that the preserving concentration of the alcohol, taking into account the dry matter of the samples, varied between 70 and 80 per cent. Wells and Caldwell (1914) have given evidence to show that to prevent completely autolysis in blocks of liver tissue requires an alcohol concentration of at least 90 per cent. With alcohol strengths of 80 per cent or over, he found only a slight degree of autolysis, but below that concentration the autolytic action was distinctly measurable. In the light of their experiments it would seem that slight autolysis might be expected in these muscle samples. However, the alcohol concentrations approached the limiting concentrations advised by Wells and Caldwell and the tissues were finely ground, quickly and intimately mixed, and heated somewhat during the sealing, all of which favor the inhibition of autolysis. Furthermore, Hoagland, McBryde, and Powick (1917) have recently reported the changes during the aseptic autolysis of beef muscle during cold storage, the most characteristic being that in the amino-acids. The amino nitrogen increased from 78 to 766 mg. per 100 gm. of fresh sample, a total increase of 840 per cent. In view of these results they suggest the study of the amino nitrogen as a sensitive indicator to the presence of autolysis in animal tissues. In the present investigation, the marked constancy of the amino nitrogen content of a series of diverse samples speaks against the presence of any such disturbing factor as autolysis. The close agreement of the total protein content with the data presented by Charles W. Greene (1919) from earlier analyses, made in 1909, is proof of the adequate preservation of the samples.

Methods of Analysis.

The analysis of the muscle was carried out according to the improved method of Janney.[3] The sample was placed in a casserole and boiled

[2] The writer is under deepest obligation to Prof. Lafayette B. Mendel or friendly advice and direction during the progress of the study.

[3] Janney, N. W., *J. Biol. Chem.*, 1916, xxv, 177.

three or four times with 50 cc. portions of alcohol. This completed the coagulation of the muscle proteins and also removed some of the fat from the tissue. The samples highest in fat received a preliminary extraction with ether, since the removal of the excess fat greatly facilitates the subsequent extraction with water.

After the coagulation with alcohol, the muscle was repeatedly extracted with boiling water, in 50 to 100 cc. portions. This water extraction was repeated seven or eight times, the total volume of the extract being between 800 and 900 cc. The protein residue was then washed onto a tared filter, the filter folded around it, and the whole extracted with 95 per cent alcohol in a Soxhlet for 3 to 6 hours. This was followed by extraction with absolute alcohol for 15 to 20 hours. Although Janney considered an ether extraction of the protein residue unnecessary, yet on account of the high fat content of these samples, a 12 hour extraction with ether was resorted to as a measure of precaution. After completion of the extraction the filter and residue were transferred to a glass-stoppered weighing bottle, dried to constant weight at 105°, and the weight of the filter was deducted.

The alcohol and water extracts were combined in a large Claisen flask and evaporated to dryness under diminished pressure. The extracts were transferred to a small bottle with ether and water and extracted in a Soxhlet with ether for 15 hours, as suggested by Saiki.[4] This separation of the ether- and water-soluble extractives was not entirely satisfactory. It seemed, however, to be preferable to the acid-chloroform-water method of Koch.[5] The greatest difficulty occurred in the recovery of the water-soluble material carried over into the ether. Recovery was attempted by shaking out the ether extract, with water, in a separatory funnel. Owing to extensive emulsion formation, this was not a success. The procedure finally adopted was to evaporate the ether extract and redissolve in a minimum of anhydrous ether. The solution was filtered, the ether distilled off, and the semipurified ether extract dried to constant weight at 100°, as provided by the official methods of analysis.[6]

That portion of the crude ether extract insoluble in anhydrous ether was dissolved in hot water and returned to the water extract. When the original alcohol-water extract was concentrated, a small amount of insoluble material separated. This was filtered off, dried, and weighed. The weight of this recovered material was then added to that of the original protein residue, to secure the total.

The filtered solution of the water-soluble extractives was transferred to a volumetric flask and made up to a definite volume, usually 300 cc. To avoid the possibility of change during drying, this water extract was divided into aliquots. One of these, of 100 cc., was evaporated on a water bath and dried to constant weight at 105°, an aluminum capsule with a

[4] Saiki, T., *J. Biol. Chem.*, 1909–10, vii, 21.
[5] Koch, W., *J. Biol. Chem.*, 1907, iii, 159.
[6] *U. S. Dept. Agric., Bull. 107*, 1909.

closely fitting lid being used as a container. The weight of the total solids in the water extract was then calculated.

The weight of the total solids in the muscle was taken to be the sum of the weights of the insoluble residue, the ether-soluble, and the water-soluble materials. In their first paper, Janney and Csonka[7] mentioned that the results obtained in this way were reasonably accurate. They recovered between 99.7 and 100.6 per cent of the amounts found by direct determination. The water was taken as the difference between the weights of the fresh sample and the total solids. Owing, therefore, to losses in the manipulation of the other materials, the water determinations should show the greatest error.

The total extractive nitrogen was determined in an aliquot of the water extract. The distillation method of Bock and Benedict[8] was used and the ammonia in the distillate not Nesslerized but titrated with 0.5 N acid, as suggested by Folin.[9] The amino-acid nitrogen in the water extract was determined in a 150 cc. portion by the micro apparatus of Van Slyke.[10] In the removal of the urea and residual traces of protein, urease and trichloracetic acid were used, according to the method chosen by Bock,[11] for blood. The creatine in the water extract was determined by the procedure of Janney and Blatherwick.[12] Because of its greater convenience the Myers autoclave method of conversion was employed in place of heating on the water bath. Janney and Blatherwick found this method accurate when used on muscle extracts, though it gave erroneous results with other tissue extracts. In order to reduce to a minimum the errors in the colorimetric reading, varying amounts of extract were taken, so that the reading of the unknown and the standard were always approximately equal.[13]

A general table (Table I) is presented giving the grand totals of the separations. This table is valuable chiefly as a background for the special study of nitrogenous extractives. However, attention is called to the very close agreement with the earlier analyses of Charles W. Greene on this series of fishes. His analyses are based primarily on Koch's methods, mine on Janney's—both modified in adaptation to the salmon muscle tissue.

[7] Janney, N. W., and Csonka, F. A., *J. Biol. Chem.*, 1915, xxii, 195.

[8] Bock, J. C., and Benedict, S. R., *J. Biol. Chem.*, 1915, xx, 47.

[9] Folin, O., *J. Biol. Chem.*, 1915, xxi, 195.

[10] Van Slyke, D. D., *J. Biol. Chem.*, 1913–14, xvi, 121.

[11] Bock, J. C., *J. Biol. Chem.*, 1916, xxviii, 357.

[12] Janney, N. W., and Blatherwick, N. R., *J. Biol. Chem.*, 1915, xxi, 567.

[13] The picric acid was purified as suggested in Folin, O., and Doisy, E. A., *J. Biol. Chem.*, 1916–17, xxviii, 349.

TABLE I.

Changes in Composition of Muscle of Salmon during Migration.

Station.	Salmon No. and sex.	Water.		Total solids.		Protein residue.			Water-soluble extractives.			Ether extract.	
		Fresh.	Fat-free.	Fresh.	Fat-free.	Fresh.	Dry.	Fat-free.	Fresh.	Dry.	Fat-free.	Fresh.	Dry.
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Ilwaco, Wash.	1276♂	62.2	75.0	37.9	25.0	17.1	45.3	20.7	3.60	9.5	4.34	17.1	45.2
Tidewater.	1279♀	62.6	74.2	37.4	25.8	17.2	46.0	20.4	4.55	12.1	5.39	15.6	41.8
	1281♀	61.6	75.4	38.4	24.6	16.3	42.4	19.9	3.78	9.9	4.63	18.3	47.7
Average		62.1	74.9	37.9	25.1	16.9	44.6	20.3	3.98	10.5	4.79	17.0	44.9
Warrendale, Ore.	1259♂	60.3	75.4	39.6	24.6	15.9	40.1	19.9	3.79	9.6	4.73	20.0	50.3
130 miles.	1261♀	63.7	75.6	36.3	24.4	16.6	45.7	19.7	4.00	11.0	4.75	15.1*	43.3
	1262♂	61.4	75.8	38.6	24.2	15.9	41.3	19.7	3.68	9.5	4.54	19.0	49.2
	1263♀	65.5	74.9	34.5	25.1	17.9	52.0	20.5	4.01	11.6	4.59	12.6	36.4
Average		62.7	75.4	37.3	24.6	16.6	44.8	19.9	3.87	10.4	4.65	16.8	44.8
Seufert, Ore.	1040♂	65.0	75.9	35.0	24.1	16.7	47.7	19.5	3.95	11.3	4.61	14.4	41.1
210 miles.	1056♀	62.5	75.1	37.5	24.9	16.5	44.1	19.9	4.16	11.1	5.00	16.8	44.8
	1060♀	65.4	75.8	34.6	24.2	16.6	47.9	19.3	4.19	12.1	4.86	13.9	40.2
	1074♂	62.5	75.7	37.5	24.3	16.5	44.0	20.0	3.57	9.5	4.32	17.4	46.5
Average		63.8	75.7	36.2	24.3	16.6	45.9	19.7	3.96	11.0	4.70	15.6	43.2

Ontario, Ore.	1312♀	70.1	76.7	29.9	23.3	17.2	57.5	18.8	4.15	13.9	4.53	8.6	28.6
700 miles.	1314♂	67.8	76.4	32.2	23.6	16.3	50.5	18.3	4.72	14.6	5.31	11.2	34.9
	1317♂	70.9	76.8	29.1	23.2	17.3	59.6	18.8	4.08	14.0	4.41	7.7	26.3
	1321♀	70.9	77.7	29.9	22.3	15.7	52.5	17.4	4.25	14.2	4.71	9.8	32.7
Average		69.7	76.9	30.3	23.1	16.6	55.0	18.3	4.30	14.2	4.75	9.3	30.6
Cazadero, Ore.	1295♂	78.6	80.9	21.4	19.1	14.5	67.6	14.9	4.05	18.9	4.16	2.9	13.6
Spawning grounds.	1296♀	79.4	83.0	20.6	17.0	12.9	62.7	13.4	4.03	19.5	4.18	3.7	17.7
	1299♀	79.4	82.7	20.6	17.3	12.2	59.0	12.7	4.37	21.2	4.55	4.1	19.8
	1300♂	78.5	81.2	21.5	18.8	13.8	64.4	14.3	4.30	20.0	4.45	3.3	15.6
Average		79.0	82.0	21.0	18.0	13.3	63.4	13.8	4.18	19.9	4.34	3.5	16.7

* The original analysis was lost. The results of Charles W. Greene are given here.

Chemical Changes in Extractives of Muscle.

Hatai (1917) found that in the white rat the extractives in the muscle varied with the amount of protein, and were, he considered, an index to the activity of the tissue. The same seems to be true of the salmon muscle (Table II). The average figure for the extractives in the fat-free tissue for the samples from Ilwaco is undoubtedly too high, owing to suspected error in the analysis of one of the samples (No. 1279, Table I). The figure for the corrected average is probably not much over 4.5 per cent. There is a slight increase in the muscle extractives of the fishes obtained through the successive stations as far as Ontario and a very slight decrease to the amount present at Cazadero. The variation in

TABLE II.

*Changes in Composition of Muscle of Salmon During Migration.**

Station.	Location.	Protein residue.	Water extract.	Extractive nitrogen.	Amino nitrogen.	Total creatine nitrogen.
		per cent	*per cent*	*per cent*	*per cent*	*per cent*
Ilwaco	Tide-water.	20.3	4.79	0.482	0.061	0.081
Warrendale	130 miles.	19.9	4.65	0.524	0.076	0.094
Seufert	210 "	19.7	4.70	0.521	0.078	0.078
Ontario	700 "	18.3	4.75	0.553	0.074	0.089
Cazadero	Spawning grounds.	13.8	4.34	0.534	0.084	0.089

* Averages in percentages as calculated on the fat-free basis.

the amount of extractives present in the muscle, therefore, parallels the activity of the catabolic processes in the salmon in somewhat the same manner in which it parallels the anabolic processes in the growing rat.

Total Extractive Nitrogen.

The total extractive nitrogen shows variations comparable with those in the total extractives. Charles W. Greene (1919) found that the ash content of the muscle was relatively constant, or else slightly decreased during the migration. With this in mind, it becomes evident that the extractive nitrogen remains approximately constant in its relation to the extractives of the muscle.

Phylogenetically it is of interest to note that the percentage of extractive nitrogen in the salmon muscle (Table III) is very much higher than in mammalian tissue. The figures are practically identical with those given by Buglia for other teleosts, and by Wilson for some invertebrates. It should be noted that among the invertebrates Buglia found much more extractive nitrogen than did Wilson. They analyzed different species, however, so that much emphasis cannot be placed upon this difference.

TABLE III.

Total Extractive Nitrogen.

Station.	Location.	Fat-free sample.	Water extract.	Protein residue.	Water in muscle.
		per cent	*per cent*	*per cent*	*per cent*
Ilwaco	Tide-water.	0.482	10.1	2.37	0.708
Warrendale	130 miles.	0.524	11.2	2.63	0.694
Seufert	210 "	0.521	10.8	2.67	0.689
Ontario	700 "	0.553	11.7	3.04	0.670
Cazadero	Spawning grounds.	0.534	12.6	3.82	0.652

Amino Nitrogen.

The salmon muscle is unique in the extent and character of the protein utilization during the spawning migration. Great interest, therefore, attaches to the amino-acids at known stages in the process of protein decomposition. The amino nitrogen in the muscle shows a striking increase from 61 to 84 mg. per 100 gm. of fat-free tissue, or 39 per cent (Table IV). This increase is not confined to the muscle alone, but also includes the

TABLE IV.

Non-Protein Amino Nitrogen.

Station.	Location.	Fat-free sample.	Water extract.	Protein residue.	Water in muscle.
		per cent	*per cent*	*per cent*	*per cent*
Ilwaco	Tide-water.	0.061	1.280	0.300	0.082
Warrendale	130 miles.	0.076	1.640	0.380	0.101
Seufert	210 "	0.078	1.660	0.400	0.098
Ontario	700 "	0.074	1.520	0.410	0.096
Cazadero	Spawning grounds.	0.084	1.860	0.590	0.099

amino nitrogen in proportion to the rest of the extractives, indicating that the amino-acids are, perhaps, more active during the migration than are the extractives as a whole.

Particularly striking is the enormous increase in the amount of amino-acids relative to the protein from which they must originate. The amino nitrogen in the fat-free muscle increases by over 35 per cent. At the same time the protein content of the muscle decreases by nearly the same amount (Greene, 1919). In relation to the protein the increase in the amino nitrogen is, in round numbers, 100 per cent. Evidently the amino-acids are liberated at the expense of an ever increasing wastage of the muscle substance.

The extractive nitrogen shows an increase of 33 per cent in the amount present relative to the protein content of the muscle. Though this increase is not so extreme as that of the amino nitrogen, it alone is sufficiently striking to emphasize the great exaggeration of the protein catabolism occurring in the salmon during the migration fast.

While emphasis is laid on the fact of the increase in the amino-acids at the expense of the muscle protein, even more importance is to be attached to the effect of the influx of water into the tissue. The increase in the water content of the muscles during migration has already been pointed out by Greene (1915), also previously in this paper. The amino nitrogen relative to the water present in the tissues increases from 80 to 100 mg. per 100 gm. of water at the very beginning of the migration and remains unaffected by subsequent changes in the muscles. With the stimulated protein catabolism during the migration fast, the muscles and particularly the fluid in the muscles become saturated with amino nitrogen early in the migration period. This saturation then continues to the end (Table IV). The limiting factor in the behavior of the amino-acids would seem to be the volume of water present in the tissue, and not the protein content.

The changes here observed in the amino nitrogen content of the salmon lend a new significance to, and afford an illuminating extension of, the studies of Van Slyke and Meyer. It is to them that we are at present largely indebted for our knowledge of the behavior of the amino-acids in the body after absorption from the intestine. Van Slyke and Meyer (1912) demonstrated

that in dogs the amino-acids are absorbed into the blood stream from which they rapidly disappear as the blood circulates through the tissues. Their experiments have been confirmed by Delaunay (1913) and others. Van Slyke and Meyer (1913–14) have further shown that this disappearance from the blood stream is due to the absorption of the amino-acids by the muscles.[14] They write as follows:

"The disappearance of the intravenously injected amino-acids from the circulation is the result of neither their destruction, synthesis, nor chemical incorporation into the cell proteins. The acids are merely *absorbed from the blood by the tissues, without undergoing any immediate chemical change*. In the case of the muscles at least, a fairly definite saturation point exists, which sets the limit to the amount of amino-acids that can be absorbed. We have never been able to force the amino nitrogen figure of the striated muscles above 75-80 mg. per 100 grams. The capacity of the internal organs is more elastic; we have raised the amino figure of the liver to 125–150 mg.

"The absorption of amino-acids from the circulation by the tissues, although extremely rapid, is never complete; the blood contains 3–8 mg. of amino-acid nitrogen per 100 cc. even after a fast of several days' duration. The amino-acids of the blood appear, therefore, to be *in equilibrium* with those of the tissues, a condition which accounts for all the observed phenomena, and would also account for any transfer of amino-acids which may occur from organ to organ, or from maternal organs to fetus.

"The process by which the amino-acids are taken up and held by the tissues cannot be wholly osmotic, because the normal concentration of the amino nitrogen in the tissues is five to ten times that in the blood; and even when the latter is suddenly loaded with injected amino-acids, they quickly gather in not equal, but greater, concentration in the tissues. The most probable explanations of the process are, that it is either; (1) a mechanical absorption, or (2) the formation of loose molecular compounds between the amino-acids and the tissue proteins, such as Pfeiffer has recently shown can be formed by the amino-acids themselves with inorganic salts."

Van Slyke and Meyer found a varying behavior on the part of the amino-acids after absorption by the tissues. They disappeared rapidly from the liver, with a simultaneous increase in the urea content of the blood indicating that the liver was able to metabolize the absorbed amino-acids. The muscles on the contrary showed but little such power, and the absorbed amino-acids were lost but slowly.

[14] Van Slyke, D. D., and Meyer, G. M., *J. Biol. Chem.*, 1913–14, xvi, 212.

Van Slyke and Meyer (1913–14, *c*) in continuation of their investigations found that the amino-acid content of the muscles of dogs was not affected by heavy protein feeding, but did increase upon fasting. These writers summarize their views in the following manner.[15]

"The amino-acids appear, therefore, to be intermediate steps, not only in the synthesis, but in the breaking down of body proteins. Otherwise, in order to explain their maintenance in the tissue during starvation, one would be forced, contrary to the conclusions of all experimental work on the subject, to assume that they are inert substances, lying unchanged for long periods, even when most urgently needed to build tissue or supply energy. The maintenance of the amino-acid supply by synthesis, from ammonia and the products of fats or carbohydrates, seems excluded. The supply of raw material in the form of fat and carbohydrates nearly disappears during starvation, and the ammonia could originate only from broken-down protein, as the normal store of ammonia nitrogen is only a fraction of that of the free amino-acids. These considerations, and the self-evident wasting of starved tissues, point strongly to autolysis as the main source of the free amino-acids in the fasting body."

The changes in the muscles of the salmon during the fast of the spawning migration furnish clear-cut and uncomplicated evidence that the muscle tissue is the source of the free amino-acids in the fasting body. This is independent evidence confirmatory of that quoted above and derived by Van Slyke and Meyer from studies on fasting dogs. Furthermore, we emphasize the fact that in the salmon a mechanism is present capable of maintaining the amino nitrogen at a constant level of saturation in the water present in the tissues, even though this water increases in amount during the fast. This saturation is kept up regardless of the great loss in muscle protein until, in the end, the exhaustion of the organism proceeds to a degree culminating in death. It is evident that this previously undescribed water-volume effect is a strong regulating factor and must be added to those suggested by Van Slyke as probably controlling the amino-acid content of the tissues.

Creatine Nitrogen.

The figures obtained for the total creatine nitrogen show a very great amount of variability (Table V). That the variations

[15] Van Slyke, D. D., and Meyer, G. M., *J. Biol. Chem.*, 1913–14, xvi, 232.

cannot entirely be charged to manipulation of samples is shown by the fact that the amino nitrogen determinations underwent nearly the same manipulation and yet they gave consistent results. Some other undiscovered factor was involved. No effort was made to determine the creatine and creatinine separately.

The creatine nitrogen in percentage of the fat-free muscle revealed no constant variation in relation to the fast. The variability of the analyses was sufficient to mask any slight degree of change. Certainly, there was no evidence of any such enormous lowering of the creatine content of the muscle as that described by Howe and Hawk (1911) in fasting dogs. Instead of decreasing, *the creatine in the salmon muscle increased in proportion to the protein residue.* This increase is definite and above the influence of possible variations in the muscle samples.

TABLE V.

Total Creatine Nitrogen.

Station.	Location.	Fat-free sample.	Water extract	Protein residue.	Water in muscle.
		per cent	*per cent*	*per cent*	*per cent*
Ilwaco	Tide-water.	0.081	1.720	0.400	0.108
Warrendale	130 miles.	0.094	2.030	0.470	0.125
Seufert	210 "	0.078	1.690	0.400	0.103
Ontario	700 "	0.089	1.880	0.510	0.115
Cazadero	Spawning grounds.	0.089	1.930	0.610	0.106

Unfortunately no metabolism studies in fishes, involving complete examination of the excreta, are available as a means of throwing light upon the behavior of the creatine during the breaking up of the muscle. The further study of creatine metabolism in the salmon should be of marked interest in the light of the present views of Thompson (1917, *a*, *b*) regarding the probable metabolic relationship of arginine and creatine, and of the heightened arginine metabolism in the salmon incident to the development of the reproductive organs. The material available in the present instance was not sufficient to permit of such investigation.

The present studies are inadequate to decide whether the relative constancy of the muscle content of creatine nitrogen

is due to the liberation of increased amounts proportionate with the disintegration of the muscle protein, or to the maintenance of a constant saturation of the muscle fluids, as is true of the amino-acids.

General Significance of the Changes in the Muscle of the Salmon during the Spawning Migration.

The mechanism concerned in the transference of material in the salmon is still unsettled. Miescher (1897) interpreted the changes he observed as being a fatty degeneration associated with "liquefaction" of the muscle. The cause of this "liquefaction" Miescher associated with the increased vascularity of the gonads and a reduced flow of blood to the muscles. The resulting insufficient tissue respiration he believed to be the primary cause of the breakdown of the muscles. It is not necessary to discuss the details of the method of protein transportation hypothecated by Miescher. They were based upon Voit's idea of organized and circulating protein, which is now abandoned in view of the present day conception of the rôle of the amino-acids in protein metabolism.

In his critique of Miescher's theory, Paton says:[16]

"The theory, however, assumes that the change in the muscle is a degeneration, which it is not, and it affords at best but a partial explanation of the condition. It is too mechanical, and leaves unsolved the problem of what starts the growth of the ovaries, what causes the dilation of the blood vessels there, and thus leads to the diminished blood supply to the muscles. The growth of the ovaries may be considered a cyclic function, but in all these cyclic functions the nervous system is intimately involved. It is well known that not only is the blood supply to every part of the body under the control of the nervous system, but that the very rate of chemical change in the cells of the body is also under the influence of the nerves. Not only does the brain bring about and control the extensive chemical changes in the muscles which lead to movements, but it also governs the slower chemical changes, such as those by which heat is produced in the warm blooded animals. The building up and breaking down processes are alike controlled by nerves, and it is only fair to assume that the growth of the ovaries and testes and the discharge of material from the muscle for their growth are primarily determined by the nervous system, and that the vascular changes are secondary and not causal. In this con-

[16] Paton, D. N., *Report Fishery Board of Scotland*, 1898, iv, 175.

nection it must be remembered that throughout the whole period the muscles remain active, and not only excrete material to the ovaries and testes but also set free energy of the proteids and fats stored within them, a state of matters irreconcilable with the idea of the existence of a degenerative process."

In this connection, reference should also be made to the histological studies of Charles W. Greene (1913) upon the behavior of the fats in the muscle of the Columbia River salmon. He points out and emphasizes that not only the intermuscular but also the intramuscular fat is storage fat and that the muscles are the great fat storage organs of the salmon. Regarding the smaller amounts of intramuscular fat present, he says:[17]

"The salmon muscle fat is a filtration fat, not a fatty degeneration. It may be stated here that the studies on the king salmon tend to disprove Miescher's theory that the intracellular fat of the salmon muscle, of whatever type the muscle, is a fatty degeneration, a 'Fettentartung;' and support the observations of Mahalanobis that the process is an 'infiltration.' In short, the observations made on the king salmon have tended to confirm the view . . . . that the intracellular fat of the king salmon is an expression of the nutritive state of the muscle. It is a loading of fat by a process of infiltration, ... and is not a degeneration of the muscle substance."

Under the topic "The mechanism of fat transference in the salmon body" Greene further advocates the view that fat transformations and transferences are under enzymatic regulation, that the whole process is in this case a function of lipase reactions, in short, is governed by chemical factors.

To extend this view to the field presented here would lead us to advocate that chemical factors govern the regulation of the protein changes during the migration, as well as the fat changes. Undoubtedly the breakdown of the muscle is autolytic in nature.

Bradley and Taylor (1916) have reemphasized the fact that autolytic processes are very sensitive to the reaction, being markedly accelerated by slight increases in the hydrogen ion concentration of the medium. On this basis they explain the postpartum involution of the uterus as due to autolysis. This autolysis is favored by vascular changes in the organ which may

[17] Greene, Chas. W., *U. S. Bureau of Fisheries Bull.*, 1913, xxxiii, 123.

result in the development of a local acidosis. A similar set of changes can scarcely determine the autolytic processes in the salmon muscle. Greene (1904) found that in the Pacific salmon the blood pressure was maintained at its original high level even at the end of the migration. The muscles are also exceedingly active through the whole migration period, and the oxidations therein must be correspondingly intense. Certainly there is no evidence of "Zenker's degeneration" such as Wells (1914) produced by the action of acids or by overstimulation of the muscle.

The true factor regulating the catabolism of the muscle protein is probably the energy requirement of the salmon. Fat is the chief and primary source of energy during the spawning migration. As migration proceeds, however, more and more of the protein as well is catabolized. Protein is also consumed in the development of the growing genitalia. According to the present day theories of metabolism this protein cannot be catabolized directly, but only after breaking down into the so called "building stones." The muscle shows a remarkable constancy in the relative amounts of extractives present during the migration. Particularly constant is the concentration of the extractives in the water contained in the protoplasm. Van Slyke and Meyer (1913–14) have shown that an equilibrium exists between the amino-acids of the muscles and blood, and it is possible that similar equilibria exist for others of the muscle extractives. With the removal of amino-acids from the muscles or blood either by synthesis into testicular or ovarian tissue, or by oxidation for the production of energy, these particular equilibria are disturbed. This is the state in which more of the tissue protein is broken down to bring the amino-acid content of the muscles back to the normal level.

Physiologically the changes in the muscles of the salmon during the fast of the spawning migration are most interesting. Ordinarily, in mammals especially, fats and carbohydrates form the greater part of the energy-producing substances in the body. In the salmon, on the other hand, fats and proteins are the sole energy-producing substances. Charles W. Greene (1913) has found that carbohydrate does not enter into the physiological economy of these fish, and has followed the course of the fats, showing their great importance as a source of energy during the migration. He also has laid stress on the significance of the great decrease

in the amount of protein arguing that this is stored protein necessary for the maintenance of muscular and other activity in the fasting salmon.

In the present paper, evidence has been presented for the presence in the fasting salmon of a regulatory mechanism that maintains the concentration of amino nitrogen in the muscle fluids at an approximately constant level. The significance of such a mechanism for the control of protein metabolism in general is obvious.

SUMMARY.

The results of this investigation that call for especial emphasis may be stated as follows.

1. A table giving the percentage composition of salmon muscle from the different stations is presented which confirms by a different method the determinations of Charles W. Greene and extends the analyses to the separation of the non-protein nitrogen fractions.

2. The total extractives in the fat-free tissue increase slightly during the greater part of the migration. The amount of extractives in muscles seems, therefore, to be an index of the metabolic activity of these tissues. This is true both for the tissues of the growing rat as studied by Hatai, and for those of the fasting salmon studied in the present instance.

3. The percentage of nitrogen in the organic extractives remains approximately constant during the migration period. The changes in the extractive nitrogen are, therefore, the same as those in the extractives as a whole.

4. The non-protein amino nitrogen increases in percentage in the fat-free muscle during the migration. The increase in the amount of amino nitrogen relative to the protein from which it must be formed is especially marked, being nearly 100 per cent.

5. The concentration of amino nitrogen relative to the water in the muscles increases from 82 to 100 mg. per 100 gm. of water coincidently with the augmentation of catabolism at the beginning of the migration. The amino nitrogen then shows no further change during the migration. The amount of amino-acids in the tissue is dependent, therefore, upon the volume of water present. This water-volume effect must be added to those factors suggested by Van Slyke as controlling the amino-acid content of the tissues.

6. The creatine nitrogen determinations were rather variable. In general it may be stated that the total creatine content of the fat-free muscle is not essentially changed during the migration fast. Relative to the protein in the muscle the creatine nitrogen increases sharply.

7. The presence in the body of a mechanism regulating the concentration of amino nitrogen in the fluids of the tissues is indicated. The importance of this observation and its significance relative to the theories of protein metabolism are discussed.

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DIRECT DETERMINATION OF NON-AMINO NITROGEN IN THE PRODUCTS OF PROTEIN HYDROLYSIS.

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In the previous method used by Van Slyke[1] for analyzing proteins according to the chemical groups characteristic of the different amino-acids, the non-amino nitrogen was determined indirectly by calculating the difference between the total nitrogen of the amino-acids, estimated by Kjeldahl and the amino nitrogen, estimated by the Van Slyke method. As results thus calculated are subject to the added errors of the two determinations, an attempt has been made to devise a method by which the non-amino nitrogen could be estimated directly from a single determination.

Principle of the Method.

The procedure is exactly the same as that used in the former method for protein analysis[1,2] until the hexone bases have been separated from the remaining amino-acids. At this point we turned our attention to the filtrate from the bases.

Since the bases are precipitated with phosphotungstic acid, the filtrate contains the excess of this acid as well as that used in the washings. It was found necessary to remove all traces of the phosphotungstic acid before proceeding, as its presence interferes with the reduction of nitrites by the zinc-copper couple which occurs later in the procedure.

In the estimation of the total nitrogen of the filtrate and the amino nitrogen, however, it is important that the determination be performed before removal of the phosphotungstic acid, because the amyl alcohol-ether mixture which we use for extracting the phosphotungstic acid[2] also extracts some of the amino nitrogen,

[1] Van Slyke, D. D., *J. Biol. Chem.*, 1911–12, x, 15.

[2] Van Slyke, D. D., *J. Biol. Chem.*, 1915, xxii, 281.

as is shown by Table V. Peculiarly, it does not extract any of the non-amino nitrogen.

After the removal of the phosphotungstic acid with amyl alcohol and ether, the solution is ready to use for the determination of the non-amino nitrogen. This was accomplished in three steps.

1. The Removal of Primary Amino Nitrogen by Reaction with Nitrous Acid.—$RNH_2COOH + HNO_2 = N_2 + H_2O + ROHCOOH$. In order to reduce the amount of inorganic nitrogen introduced to a minimum small amounts of sodium nitrite are used for the production of nitrous acid, and the reaction is accelerated by heat instead of by a large excess of reagents.

At first small amounts of glacial acetic acid were used for the production of nitrous acid. On testing out the method on a solution of proline, the results were found somewhat high. That this error was not due to an incomplete removal of the amino nitrogen is shown by Table I. That it was not due to an incomplete reduction of the nitrite is shown by Table II. A further test was made by varying the amount of proline used for testing out the method. It was found that the increase in nitrogen over that calculated was directly proportional to the amount of proline used. This is shown in Table III. It seems that there is a fixation of some of the nitrite nitrogen presumably attached to the proline as nitroso-nitrogen, $RNH + HNO_2 = RN - NO + H_2O$. In order to break up such compounds hydrochloric acid was substituted for acetic, and theoretical results were obtained.

2. The Removal of Excess Nitrous Acid.—The nitrogen of the nitrous acid which reacts with NH_2 groups escapes as N_2 gas, but some of the excess nitrous acid necessary for the completion of the reaction remains. This must be quantitatively removed before the residual non-amino organic nitrogen can be determined by Kjeldahl. Several methods were tried, but the only one which gave quite satisfactory results was reduction to ammonia by the zinc-copper couple. The technique used by Scales[3] was adapted to our purpose. The reduction is carried out in a boiling solution made alkaline with magnesium oxide, so that the ammonia can be distilled off as formed.

3. The Determination of the Residual, Non-Amino Nitrogen.—This was done by the ordinary Kjeldahl method.

[3] Scales, F. M., *J. Biol. Chem.*, 1916, xxvii, 327.

Details of Procedure in Protein Analysis.

The filtrate from the hexone bases,[1,2] representing 2 or 3 gm. of protein, is concentrated according to the original method of Van Slyke[1] to a volume of 150 cc. Portions of this solution in our experiments have been used for determination of the total nitrogen and amino nitrogen as described in Van Slyke's original paper. A portion of the remainder was freed of phosphotungstic acid and used for determination of non-amino nitrogen as described below.

Total Nitrogen in the Filtrate from the Bases.—10 cc. portions were taken for Kjeldahl, using 10 gm. of potassium sulfate, 0.2 gm. of copper sulfate, and 20 cc. of concentrated sulfuric acid.

Amino Nitrogen in the Filtrate from the Bases.—2 cc. portions were used for the amino determination, using the micro-apparatus in the usual manner with a 5 minute reaction period.

Direct Determination of Non-Amino Nitrogen in the Filtrate from the Bases.—Of the 150 cc. of filtrate 100 cc. are measured into a separatory funnel of 500 cc. capacity. 5 cc. of concentrated hydrochloric acid and 75 cc. of a mixture of equal parts of amyl alcohol and ether are added. The funnel is shaken 1 or 2 minutes, and the aqueous layer separated. The extraction is repeated with three more successive portions of amyl alcohol-ether, using 50, 25, and 25 cc. for the successive extractions. Finally the combined amyl alcohol-ether extracts are shaken out once with water to remove traces of water solution which might have been carried along. This washing is extracted once with fresh amyl alcohol-ether, and combined with the main solution. Solutions thus treated were found free of phosphotungstic acid when tested with barium hydroxide. The solution is concentrated in vacuum to a volume of about 50 cc. and washed into a 100 cc. volumetric flask, thus making the solution to the original volume of filtrate taken. The results of this treatment on the non-amino nitrogen are shown in Table IV. In proteins where the non-amino nitrogen is very low, the volume could be made smaller. In case a heavy white precipitate appears on concentration, and does not go into solution on diluting and shaking, the solution is filtered after diluting to the required volume.

20 cc. portions of the solution are used for the non-amino nitrogen determination. Each portion is pipetted into a small

Kjeldahl flask to which 1.2 cc. of 30 per cent sodium nitrite and 5 cc. of concentrated hydrochloric acid (sp. gr. 1.2) are added. The flasks are suspended in a boiling water bath for 45 minutes. The solution is then neutralized to alizarin with 10 per cent sodium hydroxide.

While this reaction is taking place, the zinc-copper couples may be prepared as described by Scales.[3, 4] The neutralized solution from which the nitrite nitrogen is to be removed is washed quantitatively on to the couple, and then about 0.75 gm. of magnesium oxide is added, the sides of the flask are washed down, and the volume is made up to 200 or 250 cc. The solution is now boiled gently for 45 minutes in a hood with a good draught. The reaction is completed and the ammonia removed in this time, as is shown by Table II.

After the completion of the reaction, the solution is immediately washed from the couple into a clean dry 500 cc. Kjeldahl flask, washing the zinc four times with distilled water. The zinc is covered with water until ready for the next determination. The solution is treated with 10 gm. of potassium sulfate, 0.2 gm. of copper sulfate, and 20 cc. of concentrated sulfuric acid, boiled down, and digested for 1 hour after it clears.

Purity of Reagents.—Every reagent used must be carefully tested by blank analyses, and the correction ascertained must be applied to each determination. The phosphotungstic acid is purified with ether and water.[5] The zinc seems to be the most uncertain of all the reagents, and the most likely to cause error.

[4] Sheet zinc of about 0.5 or 0.25 mm. thickness is cut into strips 2 cm. by 8 cm. These strips are coiled by rolling loosely around a pencil, or better, with a pair of large forceps. 80 gm. of these coils are dropped into a previously weighed 500 cc. Kjeldahl flask, so that the weight of the flask plus coils is known. When the zinc is used for the first time, it is covered with an approximately 1 per cent sulfuric acid solution for 3 or 4 minutes, then a 1 per cent sulfuric acid solution for 3 or 4 minutes, until the surface is clean. In case the zinc has already been used, it is washed once with water, then covered with 1 per cent sulfuric acid until clean. The acid solution is drained off, and the zinc is covered with an acid copper sulfate solution prepared by dissolving 10 gm. of $CuSO_4.5H_2O$ in 2 liters of distilled water and then adding 6 cc. of concentrated sulfuric acid. This is allowed to stand for at least 8 minutes, until the zinc has become well coated. It is then poured off and the couple washed once with water.

[5] Winterstein, E., *Z. physiol. Chem.*, 1901-02, xxxiv, 153.

When used for the reduction of nitrites in protein digests, the zinc very soon deteriorates. For this reason it is necessary to test the couples frequently with a known proline solution, or else to renew the zinc frequently. The zinc may be used for three to six determinations, keeping the weight constant by adding more coils if necessary, but it is safest to test out the coils after the third determination. New coils should always be tested with a known proline solution before using.

EXPERIMENTAL.

To test the time required for the decomposition of amino nitrogen with nitrous acid, a 1 per cent solution of glycocoll was used. In the first three determinations, Table I, 10 cc. of a 1 per

TABLE I.

Time Required for the Completion of Decomposition of the Amino Nitrogen in a Glycocoll Solution.

Acid used.	Time of reaction.	NH_2-N found.	NH_2-N used.	Decomposition.
	min.	*mg.*	*mg.*	*per cent*
Acetic	15	0.319	18.6	98.28
"	30	0.024	18.6	99.87
"	45	0.000	18.6	100.00
Hydrochloric	30	0.412	39.2	98.95
"	45	0.000	39.2	100.00

cent solution were used, representing 18.6 mg. of amino nitrogen; in the last two, 20 cc. were used, representing 39.2 mg. of amino nitrogen, which is the maximum that is likely to occur in the amount of solution used in an analysis of protein. The glycocoll solution was measured into a small Kjeldahl flask, and 1.2 cc. of a 30 per cent solution of sodium nitrite were added, with, in the first three determinations, 0.3 cc. of glacial acetic acid, in the last two 5 cc. of concentrated hydrochloric acid. The flasks were immersed in a boiling water bath. At stated intervals flasks were removed from the bath, neutralized with sodium hydroxide, made to volume, and amino determinations were made on 2 cc. portions. The results are given in Table I.

To test the time required for the reduction of nitrite nitrogen and its complete removal as ammonia, an experiment was per-

formed using the amount of nitrite required in the method described for the direct determination of non-amino nitrogen, and proceeding with the method as described. During the reduction with zinc-copper couple the ammonia was distilled into standard acid and titrated. The results are given in Table II.

TABLE II.

Time Required for Reduction of Nitrite Nitrogen with Zinc-Copper Couple.

Time of reaction.	Nitrogen reduced.	
min.	*cc. N/14*	*mg. N*
45	33.31	33.31
	33.72	33.72
Next 15.	0.05	0.05
	0.00	0.00

The method for the direct determination of non-amino nitrogen was tested on proline and oxyproline. 20 cc. of a 0.7 per cent solution of the copper salt of proline were used, representing the maximum amount of non-amino nitrogen present in a determination as described for proteins. For the oxyproline determinations 20 cc. of a 1 per cent solution were used. In part of the determinations 0.3 cc. of glacial acetic acid was tried in place of the 5 cc. of concentrated hydrochloric acid. The results of the test and also of the use of different acids are given in Table III.

TABLE III.

Effect of Determination in Presence of Glacial Acetic and Hydrochloric Acids on Results of Non-Amino Nitrogen Determination.

Substance used.	Amount used.	Acid used.	Non-amino nitrogen.		
			Calculated.	Found.	Error.
	mg.		*mg.*	*mg.*	*mg.*
Proline	70	Acetic.	5.40	6.40	+1.0
"	140	"	10.80	12.80	+2.0
"	140	Hydrochloric.	10.80	10.73	−0.07
Oxyproline	100	Acetic.	1.27	1.52	+0.25
"	200	"	2.54	3.05	+0.51
"	250	Hydrochloric.	2.54	2.60	+0.06

A test was next made to find out the effect of treatment with amyl alcohol-ether mixture on the determination of non-amino nitrogen. A 100 cc. portion of the above mentioned proline solution was treated exactly as described above for the filtrate from the bases in hydrolyzed proteins. 10 cc. portions of the solution were used for the determinations. In determining the calculated non-amino nitrogen of the proline it was necessary to perform both a Kjeldahl and an amino determination, and then subtract the amino nitrogen ("Amino nitrogen before treatment"). This slight amount of amino nitrogen is due to traces of amino-acids present as impurities in the proline used. The latter was made from hydrolyzed protein by the ester method and purified as the copper salt, and it is difficult to free it completely from traces of other amino-acids. The results of this test are given in Table IV.

TABLE IV.

Effect of Treatment with Amyl Alcohol-Ether Mixture on the Determination of Non-Amino Nitrogen in Proline.

Total nitrogen before treatment.	Amino nitrogen before treatment.	Non-amino nitrogen.		
		Calculated.	Found.	Loss.
mg.	*mg.*	*mg.*	*mg.*	*per cent*
5.69	0.23	5.45	5.36	2.0
5.69	0.23	5.45	5.26	2.5

Casein was put through the procedure as described for proteins, and the total and amino nitrogen of the filtrate from the bases determined both before and after treatment with amyl alcohol-ether mixture. The results of the treatment are given in Table V.

TABLE V.

Effect of Treatment with Amyl Alcohol-Ether Mixture in Removing Amino Nitrogen, but not Non-Amino Nitrogen, from the Filtrate from the Bases.

Hydrolyzed Casein.

	Before treatment.	After treatment.	Loss.
	mg.	*mg.*	*per cent*
Total nitrogen	194.0	165.6	14.7
Amino "	165.0	137.0	17.0
Non-amino "			
Indirect*	29.0	28.6	1.4
Direct		28.56	1.2

* Difference between total and amino nitrogen.

TABLE VI.

Results of Analyses of Protein.

		Casein.							Gelatin.						
		Total nitrogen.	Amino nitrogen.		Non-amino nitrogen.				Total nitrogen.	Amino nitrogen.		Non-amino nitrogen.			
					Indirect.		Direct.					Indirect.		Direct.	
		gm.	*gm.*	*per cent*	*gm.*	*per cent*	*gm.*	*per cent*	*gm.*	*gm.*	*per cent*	*gm.*	*per cent*	*gm.*	*per cent*
Uncorrected	I	0.1940	0.1650		0.0290		0.0286		0.3226	0.2459		0.0767		0.0760	
	II	0.1900	0.1625		0.0275		0.0269		0.3258	0.2472		0.0786		0.0782	
Corrected for solubility of bases	I		0.1598	55.60	0.0241	8.38	0.0237	8.24		0.2407	54.40	0.0718	16.22	0.0711	16.07
	II		0.1573	54.70	0.0226	7.87	0.0220	7.65		0.2420	54.75	0.0737	16.66	0.0733	16.56
Average of corrected results.			0.1586	55.20	0.0233	8.12	0.0229	7.97		0.2414	54.60	0.0728	16.46	0.0722	16.33

Analyses of Proteins.

Casein.—12 gm. of casein were hydrolyzed with 120 cc. of 20 per cent hydrochloric acid for 46 hours, and the solution brought to 250 cc. Of this, 50 cc. portions, containing, according to Kjeldahl determinations, 0.2878 gm. of nitrogen each were used. The following determinations were made on the filtrate from the bases.

Total nitrogen, using 10 cc. portions.
Amino " " 2 " "
Non-amino " " 20 " "

The results are given in Table VI.

Gelatin.—10 gm. of gelatin were hydrolyzed with 200 cc. of 20 per cent hydrochloric acid for 40 hours, and the solution brought to 250 cc. Of this, 75 cc. portions containing, according to Kjeldahl determinations, 0.4420 gm. of nitrogen each were used. The following determinations were made on the filtrate from the bases.

Total nitrogen, using 10 cc. portions.
Amino " " 2 " "
Non-amino " " 20 " "

The results are given in Table VI.

SUMMARY.

In the mono-amino-acid fraction of the products of protein hydrolysis, as analyzed by the method of Van Slyke, the non-amino nitrogen, comprising the nitrogen of proline, oxyproline, and one-half that of the tryptophane, can be determined directly by a single Kjeldahl determination. The amino nitrogen is removed by warming with sodium nitrite and hydrochloric acid, the excess of nitrous acid is reduced with a zinc-copper couple by Scales' method, and the non-amino nitrogen in the residue determined by Kjeldahl.

The results by this method agreed closely, in analyses of casein and gelatin, with the results obtained indirectly by Van Slyke's original procedure, in which the non-animo nitrogen is calculated as the difference between total nitrogen and amino nitrogen.

It appears, therefore, that the original method, although indirect, contains no inherent errors, and that when due precautions in regard to calibrated apparatus and standardized solutions are observed, results of practically the same degree of accuracy can be obtained by the original, somewhat simpler indirect method, as by the present direct one.

THE COLORIMETRIC DETERMINATION OF HEMOGLOBIN.*

A PRACTICAL PROCEDURE.

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It is manifestly desirable for a clinical method that conditions and apparatus are made as simple as the accuracy demanded will permit. When the procedure is to be applied under field conditions, away from the conveniences of a fully equipped laboratory, this requirement for simplicity and expediency is accentuated. This communication has to do with the description of a method for accurate hemoglobin determination primarily adapted to such conditions, but equally useful in the ordinary clinical laboratory.

In 1918, Palmer (1) suggested an excellent procedure for the colorimetric determination of hemoglobin. This was essentially a modification of the method of Haldane (2), and consisted in changing the hemoglobin of the sample to carbon monoxide hemoglobin, which was compared with a standard solution. The main improvement introduced by Palmer was the use of a colorimeter to make the comparisons, which improvement added greatly to the accuracy and ease in making a determination.

Numerous tests were made in this laboratory with the Palmer method with satisfactory results; but at least one fact became evident which is at variance with the statements made by Palmer. The standards were not nearly so permanent as might be expected, though all reasonable care was taken to preserve them as recommended. The 20 per cent hemoglobin standard often deteriorated within several weeks; and the 1 per cent standard frequently

* Published with the permission of the Chief of the Section, Lt. Col. F. P. Underhill.

had to be made again every 2 or 3 days. We are unable to account for this discrepancy, except to suggest that, other things being equal, the quality of the illuminating gas used in this laboratory might be the responsible agent.

The method here described, which was designed primarily for field use, seeks to obviate the necessity for illuminating gas or carbon monoxide for reasons that are apparent. It contains no novel details, but is a new combination of previously used manipulations. The general technique recommended by Palmer is employed, but the standard solution is acid hematin as in the Sahli method (3, *a*). Further, we have adapted the method for use in the Autenrieth-Hellige colorimeter for reasons mentioned below, though other colorimeters may be used with equal facility. This method has more fully met the requirement for applicability to the routine determination of hemoglobin in dog's blood in this laboratory than has the method of Palmer.

A possible drawback to our procedure is suggested in a statement by Palmer that the colorimetric determination of hemoglobin as acid hematin cannot be used for the blood of different species. We are not aware of any experimental evidence that supports such a view. From theoretical considerations alone, there is no reason why an accurate determination by the Sahli method cannot be made upon the blood of any species of animal if the standard is at least made from the blood of that same species. Of course, whether a standard prepared from the blood of one species may be used in the measurement of the hemoglobin in the blood of another species of animal involves the question of the identity of the different hemoglobins in those physical and chemical properties utilized in the colorimetric method. As far as we know, this question remains unanswered.

The following experiment was performed to test the applicability of one standard to the bloods of different species. Cat's blood was used as the standard; and this was compared with the blood of the chicken, cat, horse, ox, rabbit, sheep, dog, and man. The comparisons were made colorimetrically by the method described in the following pages, and gasometrically by the Van Slyke method for the oxygen capacity of blood (4). The results are summarized in Table I, and show in general that the limitation placed by Palmer upon the Sahli method does not hold. In the

instances where we found a wide variation between our figures and those given by the Van Slyke method, a check by the Palmer method yielded an even more divergent result. We can offer no explanation for the few cases in which a difference occurred in the results by the Van Slyke method and ours.[1]

TABLE I.

Comparison of Hemoglobin in Different Bloods as (a) Calculated from the Oxygen Capacity, and (b) Determined Colorimetrically in the Form of Acid Hematin with One Standard (Cat's Blood).

Sample.	Hemoglobin.		Remarks.
	From oxygen capacity.	Colorimetrically as acid hematin.	
	per cent	*per cent*	
Chicken corpuscles in oxalated Ringer's solution	69.4	70.7	Turbid solution; no difficulty reading.
Defibrinated cat blood	60.0	59.3	
Chicken corpuscles in oxalated Ringer's solution	43.0	43.2	
Oxalated cat blood	91.5	91.5	
" horse "	105.0	103.0	
Horse blood + 1 per cent saline.	93.0	89.0	Checked repeatedly.
Ox "	112.0	112.0	
Defibrinated rabbit blood	93.0	95.0	Analyses made 3 days apart.
	95.8	96.7	
Oxalated cat blood	92.5	92.6	
" sheep "	60.0	59.3	
" dog "	147.0	147.8	
" human "	127.0	137.5	Checked repeatedly. By Palmer method, 145 per cent.
	126.0	141.6	
" horse "	98.6	98.4	
" dog "	91.3	92.2	
" sheep "	59.8	59.3	

[1] Berczeller (5) made a study of the use of acid hematin in the colorimetric estimation of hemolysis as found in the Wassermann reaction. From his observations upon *suspensions of red cells* he concludes that the acid hematin color is not a reliable index of the hemoglobin content in *whole blood*, because he found that lipoid material affected the color development. This conclusion does not follow from the experiments cited which apparently show that alcoholic heart extract when added to a mixture of

Procedure.

From a freely flowing source of blood, 0.02 cc. is measured by means of a calibrated Sahli pipette into 6 cc. of 0.1 N hydrochloric acid. The blood pipette is rinsed out by drawing the acid solution into it several times. Blood very low in hemoglobin may require a double sample, *i.e.* 0.04 cc. of blood in 6 cc. of acid, in order to give a dilution having the most satisfactory color for comparison with the standard. After the sample is added to the acid, the mixture must be allowed to stand, preferably in a warm place, for at least 10 minutes for the full color to develop. Readings taken sooner will be too low.

Sahli (3, *b*) claims the color development reaches a maximum in 1 minute when the proportions he recommends are used: 0.02 cc. of blood in 0.20 cc. of 0.1 N HCl.

Meyer and Butterfield (6) found considerable delay in the full development of the color of the acid hematin. We have observed that the temperature of the solution as well as the concentration of acid greatly affects the speed of color development, and have therefore resorted, when necessary, to warming the solution. An interval of 10 minutes seems to permit of practically maximum color production.

Newcomer (7) studied the rate of color development in acid hematin and found that after 10 minutes the color development was 96 per cent, after 20 minutes 98 per cent, and after 40 minutes 99 per cent of the maximum. He gives a useful formula for calculating the color deficiency: $xy = -40$, where x is the time in minutes, and y the percentage of color deficiency. The constant, -40, was apparently derived for room temperature, and is not applicable to a wide range of temperatures as our experience shows.

In cold weather, hemolysis and color development take place more slowly, and the application of gentle heat will hasten the process. As a routine procedure, immersion of the tube in a warm water bath is recommended. The color comparison may be made in either the Autenrieth-Hellige or the Duboscq colorimeter with

red cells and 0.1 N HCl causes a color development of the acid hematin more nearly approaching the maximum. In addition, it must be remembered that Berczeller worked with a tremendous disproportion of blood pigment to acid as compared with the ratios recommended by Sahli and by us. This fact evidently accounts for the inconsistencies claimed by the author.

a standard acid hematin solution. The average of at least several readings is taken. The calculation is simple and described below for each instrument.

Standard and Calculation for the Duboscq Colorimeter.—The standard for comparison is a *0.5 per cent* blood solution, which is set at 10 upon the Duboscq scale. Hence the per cent hemoglobin $= \frac{1.5 \times 10 \times 100}{\text{Reading}}$. It is desirable to make an actual calibration of the instrument for the solutions to be examined.

Standard and Calculation for the Autenrieth-Hellige Colorimeter.—The standard for comparison is a *1.0 per cent* blood solution. Experience has pointed to the necessity for different concentrations of standard solutions in each kind of instrument in order to secure most satisfactory color comparisons. In common with Palmer we have found that the scale on the Autenrieth colorimeter may be inaccurately placed. There is another source of error that cannot be corrected by a mere resetting of the scale. The glass wedge containing the standard is not mathematically perfect; therefore for accurate work, this wedge containing the 1 per cent standard should be calibrated against solutions of known amounts of blood in 0.1 N HCl contained in the small cup. Thus a curve may be constructed from which may be read at once the percentage hemoglobin corresponding to a given reading on the scale. This calibration takes a short time, and holds good for that wedge and instrument as long as other conditions are maintained.

For the conditions given here, if the instrument were perfectly constructed, the calculation would be $\frac{3.0 \times 10 \times 100}{\text{Reading}}$ = per cent hemoglobin, but this relation holds for only a portion of the scale (between 3.0 and 8.5).

Preparation of the Acid Hematin Standard.

A quantity of blood (usually 50 cc.) is obtained, carefully defibrinated, and then strained through gauze. The oxygen capacity of this blood is then determined by the Van Slyke method (4). Accepting the Haldane figure of 18.5 volumes per cent for the oxygen capacity of normal blood (corresponding to

approximately 14 gm. of hemoglobin per 100 cc.), the blood is diluted with 0.1 N HCl so as to make a 20 per cent solution of a blood with an oxygen capacity of 18.5 volumes per cent. That is, if the blood has an oxygen capacity of 18.5 volumes per cent, 20 cc. of it are diluted to 100 cc. with 0.1 N HCl; if the oxygen capacity is 22 volumes per cent then 16.8 cc. $\left(\frac{18.5 \times 20}{22}\right)$ of it are diluted to 100 cc. with the acid. The 20 per cent solution of blood thus obtained is well mixed and stored in a glass-stoppered bottle, preferably in a cool spot away from the light. This constitutes the stock solution from which the comparison standard is made. Such a stock solution will not deteriorate for at least 3 months, provided contamination by molds is avoided. Sahli (3, *b*) suggests saturating the acid with chloroform to keep molds from developing in the solution. No other unusual precautions for the preservation of this solution seem to be needed; but before using, it should be thoroughly shaken.

The comparison standard for use in the colorimeter is made by diluting 5 cc. of the stock solution to 100 cc. with 0.1 N HCl to make a 1 per cent standard; or 2.5 cc. of the stock solution to 100 cc. for a 0.5 per cent standard. Where routine determinations are to be made, it is desirable to have the standards made fresh at least once a week.

Where determinations of the oxygen capacity of the blood are not readily available, one can make the stock solution from crystallized hemoglobin.

Adaptability of Colorimeters.—The Duboscq instrument is admirably constructed and adapted to practically all colorimetric work in the laboratory. Its solidity and weight, however, militate against its ready utility in the field. We have found that comparatively inexperienced laboratory workers encounter much difficulty in making accurate color comparisons of acid hematin solutions with the Duboscq colorimeter. This difficulty, of course, became less as they gained more experience in the use of the instrument.

The Autenrieth-Hellige colorimeter embodies certain qualities that make it very desirable for this kind of work. It is light and occupies little space. The comparison standard is contained in the glass wedge which is stoppered, thus preventing variation in

the standard due to evaporation or contamination. Inexperienced workers obtain accurate readings with this instrument—perhaps because the method of making the color comparison is a more natural one. In addition, only 2 cc. of solution are necessary for making a determination in this instrument, while considerably more is needed in the Duboscq.

Accuracy of the Method.—Repeated check determinations made with the Van Slyke apparatus have demonstrated that our modification of the Sahli method as here described is accurate to within 2 per cent even in the hands of inexperienced workers. The clinical worker of experience should find no great difficulty in attaining a margin of error of 1 per cent or less.

DISCUSSION.

This method has stood the test of fairly long and intensive usage in the hands of a variety of workers with practically uniform satisfaction. It should, therefore, prove useful in the clinical and experimental laboratory because of its simplicity, general adaptability, and accuracy. A discussion such as this would not be complete without a consideration of the limitations inherent to the method. Full time must be allowed for the complete development of the acid hematin color. Any hurry in this respect will naturally endanger the accuracy of the result. Likewise, this method shares with all colorimetric hemoglobin determinations the inherent difficulties of colorimetry as pointed out by Kober (8).

On the other hand, the advantages demonstrated may be recounted as follows: (1) The method is applicable for field use because it obviates the necessity for unusual manipulations or reagents; (2) it yields accurate results in the hands of comparatively unskilled operators; (3) the standard is readily preserved for at least 3 months without undue precautions.

SUMMARY.

A method for the colorimetric determination of hemoglobin in blood is described which combines the methods of Sahli and of Palmer.

The hemoglobin of whole blood is changed to acid hematin with HCl and compared with a standard in a colorimeter. When the

Autenrieth-Hellige colorimeter is used, this method is admirably adapted for field use, and yields accurate results.

It has been shown that an acid hematin standard from the blood of one species may be used in the colorimetric determination of hemoglobin in the blood of a variety of other species of animals.

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4. Van Slyke, D. D., *J. Biol. Chem.*, 1918, xxxiii, 127.
5. Berczeller, L., *Biochem. Z.*, 1918, lxxxvii, 23.
6. Meyer, E., and Butterfield, E. E., *Arch. Int. Med.*, 1914, xiv, 94.
7. Newcomer, H. S., *J. Biol. Chem.*, 1919, xxxvii, 465.
8. Kober, P. A., *J. Biol. Chem.*, 1917, xxix, 155.

STUDIES ON PROTEINOGENOUS AMINES.

II. A MICROCHEMICAL COLORIMETRIC METHOD FOR ESTIMATING IMIDAZOLE DERIVATIVES.*

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INTRODUCTION.

The method described below is based upon the interaction between the imidazole ring and *p*-phenyldiazonium sulfonate (*p*-diazobenzene sulfonate).[1,2] Although this reaction has been used for a long time as a qualitative test for imidazoles there has been, as far as known to the authors, but one attempt made to apply it quantitatively. Weisz and Ssobolew[3] claim that a maximum color is obtained with 1 mg. of histidine dichloride under the conditions specified by them. Quantities of histidine either larger or smaller than 1 mg. give less intense colors even when the amount of *p*-phenyldiazonium sulfonate is increased. No attempt was made by the above authors to apply their method to other imidazoles. The method which we shall describe has, we believe, the following advantages over that of the above authors.

(*a*) It can be applied directly to practically any imidazole derivative.

(*b*) It gives equally good results on mixtures or pure solutions of imidazoles.

(*c*) As little as 0.00001 gm. of any of the imidazoles can be estimated with a fair degree of accuracy.

* The first article of this series was published in *J. Am. Chem. Soc.*, 1918, xl, 1716.

[1] Ehrlich, P., *Z. klin. Med.*, 1882, v, 285; *Char. Ann.*, 1883, viii, 140; *Deutsch. med. Woch.*, 1883, ix, 549; 1884, x, 419.

[2] Pauly, H., *Z. physiol. Chem.*, 1904, xlii, 508; 1905, xliv, 159.

[3] Weisz, M., and Ssobolew, N., *Biochem. Z.*, 1914, lviii, 119.

(*d*) A laborious series of dilutions is unnecessary. The first determination is usually a reliable index of the quantity of imidazole present.

In the ordinary qualitative procedure suggested by Ehrlich[1] and by Pauly[2] the imidazole solution is mixed first with the acid *p*-phenyldiazonium sulfonate reagent and this solution is then treated with an excess of a base, either sodium carbonate or ammonium hydroxide. We have found it necessary to mix the *p*-phenyldiazonium sulfonate solution first with the alkali sodium carbonate and then to add the imidazole-containing solution to this alkaline liquid to obtain strictly quantitative color productions. The necessity for such a change in procedure is readily seen when the chemical reactions involved are considered.

It is generally conceded that the first reaction proceeds according to the following equation.

(1) $HO-SO_2-C_6H_4-\underset{\underset{Cl}{|}}{NH_3}+HNO_2\rightarrow HO-SO_2-C_6H_4-\underset{\underset{Cl}{|}}{N}\equiv N+2H_2O$

By allowing a mixture containing an excess of hydrochloric and nitrous acids to react for 5 minutes at 0°, one is assured of a complete conversion of the sulfanilic acid into the diazonium salt.

After the above reaction has occurred, an excess of sodium nitrite is added to the liquid which brings about an immediate decrease in the concentration of the hydrogen ions with the formation of sodium chloride and nitrous acid.

The work of Hantzsch,[4] Goldschmidt and his coworkers,[5] von Pechmann,[6] and many others has shown that:

1. Diazonium salts are similar to ammonium salts; hence the

[4] A summary of the elaborate researches on the subject of diazonium salts may be found in Hantzsch, A., Die Diazoverbindungen, Stuttgart, 1902.

[5] Goldschmidt, H., and Merz, A., *Ber. chem. Ges.*, 1897, xxx, 670. Goldschmidt, H., and Buss, F., *ibid.*, 1897, xxx, 2075. Goldschmidt, H., and Bürkle, E., *ibid.*, 1899, xxxii, 355. Goldschmidt, H., and Keppeler, G., *ibid.*, 1900, xxxiii, 893. Goldschmidt, H., and Keller, H., *ibid.*, 1902, xxxv, 3534.

[6] von Pechmann, H., *Ber. chem. Ges.*, 1892, xxv, 3505. von Pechmann, H., and Frobenius, L., *ibid.*, 1894, xxvii, 651.

salt of a diazonium base with a weak acid, like nitrous acid, will be hydrolyzed by water to a marked extent.

2. Diazonium hydroxides pass into diazohydrates at a rapid but measurable rate of speed.

3. The speed of coupling is directly proportional to the concentration of the diazohydrate (or its nitrosamine isomer). Thus Goldschmidt and his coworkers found that the rate of coupling of diazonium salts depended upon the degree of hydrolysis (not ionization) of the salt and not upon the concentration of the diazonium salt present. Similarly the rate of coupling of the alkali diazotates was found to be proportional to their degree of hydrolysis. Goldschmidt's work does not show, however, whether it is the diazohydrate or the nitrosamine derivative that is responsible for the coupling.

4. Compounds of unquestionable nitrosamine structure such as acetylphenylnitrosamine (nitrosoacetanilide) C_6H_5-N(NO)-CO.CH_3 couple readily with aromatic amines to give diazoamino compounds and with phenols to give oxyazo compounds, acetic acid being eliminated.[6] The compounds so obtained are identical with those formed with the corresponding diazonium salts.

$$C_6H_5-\underset{\underset{NO}{|}}{N}-CO-CH_3 + H_2N-C_6H_4-CH_3 \rightarrow C_6H_5-N:\underset{\underset{H}{|}}{N}-N-C_6H_4-CH_3 + CH_3-COOH$$

$$C_6H_5-\underset{\underset{N}{\|}}{N}-Cl + H_2N-C_6H_4-CH_3 \rightarrow C_6H_5-N:\underset{\underset{H}{|}}{N}-N-C_6H_4-CH_3 + HCl$$

This fact has lead us to believe that it is the nitrosamine that is the active coupling agent and not the diazohydrate.

The series of changes that occur when an excess of sodium nitrite is added to a solution containing the diazonium salt of sulfanilic acid can be formulated as follows.

$$(2)\quad HO-SO_2-C_6H_4-N_2Cl + 2NaNO_2 \rightleftarrows \underset{(\alpha)}{NaO-SO_2-C_6H_4-N_2-O-N:O} + NaCl + HNO_2$$

$$(3)\quad NaO-SO_2-C_6H_4-\underset{\underset{N}{\|}}{N}-O-N:O + H_2O \rightleftarrows NaO-SO_2-C_6H_4-\underset{\underset{N}{\|}}{N}-OH + HNO_2$$

$$(B) \quad NaO-SO_2-C_6H_4-\underset{\underset{N=O}{|}}{N}-H \quad \rightleftarrows \quad NaO-SO_2-C_6H_4-\underset{\underset{HO-N}{\|}}{N} \quad (A)$$

$$\downarrow\uparrow \ (\beta)$$

The finished solution, after equilibrium has been established, should contain considerable of the compounds (A) and (B) either or both of which are the active coupling agents (see formulas). We have found it necessary to allow the finished diazo reagent to stand in an ice bath for at least 15 minutes before using to get uniform results.

The above solution contains variable amounts of nitrous acid, variable because some of it escapes in the form of anhydride whenever the containing flask is opened. If a substance containing an aliphatic amino group, such as histidine, is now added to the above solution, which is the qualitative procedure usually followed, variable amounts of this amino group are replaced by the hydroxyl radical. The colors produced by the hydroxy compounds are, however, not identical with those obtained from the amino compounds and, therefore, a given amount of histidine will not always give exactly the same color under the conditions prescribed for the qualitative test, since the per cent of deamination is different in each case.

If the diazo reagent were *first* mixed with an *alkali* before adding the imidazole solution, two reactions would occur: (1) the free nitrous acid would be entirely neutralized, so there could be no disruption of the aliphatic amino groups; and (2) the equilibrium represented in Equation 3 would be displaced to the right which would lead to an increase in the concentration of the active coupling compounds (A) and (B) or to the formation of the disodium salt of (A).

$$\begin{array}{l} Na-O-SO_2-C_6H_4-N \\ \qquad\qquad\qquad\qquad\quad \| \\ \qquad\qquad\qquad\qquad Na-O-N \end{array}$$

The disodium salt represented above would be rapidly hydrolyzed by water;[5] so we would not expect it to be formed in quantity unless an excess of strong alkali, such as sodium hydroxide, were added to the solution. With a properly chosen alkali, the completed solution should contain a uniform number of active coupling molecules which should then give identical colors with the same quantity of imidazole. By a strict adherence to the directions given, which were based upon the above theoretical deductions, we have found it possible to estimate imidazole derivatives with an accuracy equivalent to that of any colorimetric method.

Sodium carbonate has been found to be the ideal alkali and it was used in preference to any other because of the following facts: (*a*) with sodium acetate no color is obtained; (*b*) sodium bicarbonate gives a color only when the bicarbonate is present in large excess (even then the color appears very slowly and is lacking in intensity); (*c*) ammonium hydroxide cannot be used because, as will be shown later, it gives a green color with diazotized sulfanilic acid; (*d*) in the presence of sodium hydroxide, weak yellow to pale orange, very labile colors are produced.

In searching for a color standard against which to match the colors produced by the reaction, it was of course natural to seek first for stable, chemically allied substances. Both methyl orange and Congo red are chemically related to the colored substances produced with the imidazoles, and we have found that Congo red alone, or mixtures of it with methyl orange do indeed give the desired colors. Combinations of these indicators ought to prove useful as color standards in other cases where the colors produced are yellow to red.

EXPERIMENTAL.

Reagents Employed.

Stock Sulfanilic Acid.—Sulfanilic acid (4.5 gm.) is dissolved in 45 cc. of 37 per cent hydrochloric acid (sp. gr. 1.19) in a 500 cc. volumetric flask and water added to the mark.

Stock Sodium Nitrite.—25 gm. of 90 per cent sodium nitrite are dissolved in water and diluted to 500 cc. in a volumetric flask.

Sodium Carbonate.—Merck's or J. T. Baker's anhydrous sodium carbonate (5.50 gm.) is dissolved in water and diluted to exactly 500 cc. We specify the above two grades of carbonate because they were found to give uniform results. Two other brands tried by us were found to give yellow colors that were quite different in shade from those produced with the specified salts. The finished carbonate solution must be preserved in a glass vessel that has little tendency to dissolve in alkali. Pyrex glass vessels have proved to be entirely satisfactory.

Stock Methyl Orange.—Vacuum-dried Grübler's methyl orange (0.5000 gm.) is dissolved in water and diluted to exactly 500 cc. This solution keeps indefinitely.

Stock Congo Red.—Vacuum-dried Grübler's Congo red (2.5000 gm.) is mixed with 50 cc. of absolute alcohol in a 500 cc. volumetric flask. Water is then added to the mark. This solution keeps indefinitely.

Standard Indicator Solutions.—For the estimation of *imidazole acetic*, *imidazole propionic acid*, and *methyl imidazole*, a solution containing 1 cc. of the stock Congo red solution in a total aqueous volume of 500 cc. is employed. When this standard indicator solution has been used for comparisons the symbol (CR) is suffixed to the reading obtained.

For the estimation of *histidine* or *histamine*, 1 cc. of stock Congo red and 1.1 cc. of stock methyl orange are allowed to flow into 250 cc. of water contained in a 500 cc. volumetric flask. Water is then added to the mark. The stock indicator solutions must not be mixed in concentrated form. When this is done a rapid interaction seems to occur with destruction of color.

When this standard indicator solution has been used for comparisons the symbol (CR-MO) is suffixed to the reading obtained. When this solution is properly prepared and preserved in a tightly stoppered flask, it can be kept with certainty for 2 weeks. It is best to keep this solution in a hard glass vessel, such as Pyrex, because the alkali introduced by the solution of a soft glass changes the color of the indicators enough to give untrustworthy results.

Preparation of p-Diazobenzene Sulfonic Acid Solution. (The Reagent).—1.5 cc. each of the stock sulfanilic acid and sodium nitrite solutions are measured into a 50 cc. volumetric flask. The flask is then immersed in an ice bath for 5 minutes. Then 6 cc. more of the stock sodium nitrite solution are added and the well-mixed solution again allowed to lie in the ice bath for 5 minutes. Distilled water is then added to the mark and the flask returned to the ice bath where it is kept. This reagent must not be used for at least 15 minutes after diluting with water. We have found it to give perfect results after 24 hours. It is best, however, to prepare a fresh reagent every day.

Preparation of the Imidazoles.

Histidine Dichloride.—This was prepared according to a slight modification of the method of Fränkel.[7] Its purity was determined by analysis after it had been dried for 48 hours in vacuum.

Ammonia was absent.

It melted with gaseous decomposition at 248°.

0.06012 gm. gave 6.52 and 6.62 cc. of nitrogen gas at 20° and 750 mm., by the Van Slyke method.

	Calculated.	Found.	
	per cent	*per cent*	*per cent*
Amino nitrogen.....	6.14.	6.09	6.18

The chloride ion from 0.3009 gm. of substance was completely precipitated by 26.46 cc. of 0.1 N $AgNO_3$. This quantity of 100 per cent histidine dichloride should have used 26.40 cc.

The above two analyses prove that the histidine dichloride used was 100 per cent pure.

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[7] Fränkel, S., *Monatsh. Chem.*, 1903, xxiv, 230.

Histamine Dichloride.—The method of preparation and analysis of this compound have been previously described by us.[8]

Imidazole Propionic Acid.—Glyoxal propionic acid was prepared according to Wolff.[9] From it the imidazole propionic acid was then prepared according to Knoop and Windaus.[10] After recrystallizing the final product three times from 75 per cent acetone, too little was left to make a very exhaustive analysis. After drying in vacuum over sulfuric acid for 48 hours the pure white crystalline solid had the following properties.

It melted at 207–209°.

Ammonia was absent.

No residue on ignition.

The substance was assumed to be 100 per cent pure imidazole propionic acid.

Imidazole Acetic Acid Hydrochloride.—The substance used was obtained as a by-product in the preparation of histamine dichloride and its mode of isolation has been previously described.[11] After drying in vacuum over sulfuric acid for 48 hours the pure white, glistening solid had the following properties.

It melted at 226–228° (corrected).

Ammonia was absent.

No residue on ignition.

The chloride ion was completely precipitated from 0.1625 gm. of substance—0.10 mol—by 10.05 cc. of 0.1 N $AgNO_3$. This quantity of 100 per cent hydrochloride should have used 10.00 cc. The above properties prove that the substance used was 100 per cent pure.

4-Methyl Imidazole.—This substance was prepared from commercial glucose, ammonia, and zinc hydroxide. The directions given by Windaus and Knoop[12] were followed with the following modifications.

The solid zinc methyl imidazole hydroxide was dissolved in ten times its weight of water to which an excess of acetic acid had been added. The mixture was saturated with hydrogen sulfide under pressure and filtered from precipitated zinc sulfide. The resulting brown solution was distilled in vacuum at 50° to remove the water, hydrogen sulfide, and acetic acid. The residual dark brown gum was then extracted with ten times its weight of cold dry acetone which dissolved the methyl imidazole completely but left practically all the coloring matter behind. The oxalate was then precipitated by adding a solution of oxalic acid in acetone to the above acetone solution of methyl imidazole. The oxalate is soluble in dry acetone only in traces. The nearly white solid was then recrystallized from 75 per cent acetone until it melted at 206°. The free base was prepared

[8] Koessler, K. K., and Hanke, M. T., *J. Am. Chem. Soc.*, 1918, xl, 1716.

[9] Wolff, L., *Ann. Chem.*, 1885, ccxxix, 266.

[10] Knoop, F., and Windaus, A., *Beitr. chem. Physiol. u. Path.*, 1906, vii, 144.

[11] Hanke, M. T., and Koessler, K. K., *J. Am. Chem. Soc.*, 1918, xl, 1726.

[12] Windaus, A., and Knoop, F., *Ber. chem. Ges.*, 1905, xxxviii, 1166.

from the oxalate by the method of Windaus and Knoop, and was obtained first as a colorless mobile oil, and then as a colorless solid which had the following properties.

It melted at 56.5°.

Ammonia was absent.

No residue on ignition.

0.2000 gm. was dissolved in 5 cc. of water and titrated with 0.1 N hydrochloric acid using dimethylaminoazobenzene as indicator. Exactly 24.40 cc. of acid were required to produce the first change in the indicator which is exactly the amount demanded for this quantity of 100 per cent methyl imidazole. The substance was, therefore, 100 per cent pure.

Procedure for Estimating Imidazoles.

The method used in developing the tables as well as the general procedure for estimating imidazoles is illustrated by the following example.

$(1 - X)$ cc. of water and 5 cc. of the 1.1 per cent sodium carbonate solution are accurately measured into the right hand cylinder of a Duboscq colorimeter. 2 cc. of reagent are measured into a 5 seconds delivery 2 cc. pipette, the time noted to the second, and the reagent allowed to flow into the alkali. The contents of the cylinder are then thoroughly mixed by allowing the liquid to flow repeatedly up the inclined tube as far as safety from loss will permit. The mixing should not take over 30 seconds. X cc. of the imidazole solution are allowed to flow into the cylinder exactly 1 minute after the reagent began to mix with the alkali. The contents of the cylinder are mixed *thoroughly* as above. The test cylinder is then transferred to the colorimeter and set at 20 mm. The right hand cylinder which should contain the appropriate standard indicator solution is then adjusted constantly until a maximum reading has been obtained.[13]

The speed of color formation varies with the different imidazoles as will be made clear when the individual tables are discussed. In every case, however, a maximum color intensity is reached in the course of 5 to 10 minutes and at this point the color remains stationary for a period of from 1 to 10 minutes depending upon the imidazole used. Histamine gives a promptly appearing

[13] This is the inverse of the process as it is ordinarily carried out where the standard solution is set at some definite value and the test cylinder moved until a match is obtained.

labile color whose duration of maximum intensity is about 1 minute. With histidine the color development is gradual, takes about 6 minutes to reach its maximum intensity, and lasts from 2 to 3 minutes. In every case the weaker colors are the more stable. The most accurate determinations can be obtained by choosing such an amount of imidazole solution that the standard indicator cylinder has to be set at from 5 to 20 mm.

The method described here can be used on quantities of the imidazole-containing solution varying from 0.01 to 1.0 cc. The combined volume of water and imidazole solution used should always be 1 cc. Thus if 0.10 cc. of the imidazole solution is to be used, 0.90 cc. of water is added to the test cylinder. Then X equals 0.10 cc. and $1-X$ equals 0.90 cc.

Estimation of Small Amounts of Histidine.

A stock histidine solution was prepared by dissolving 2.000 gm. of 100 per cent histidine dichloride in water and diluting to exactly 200 cc. A layer of toluene was added as a preservative. From this the standard solution was prepared by diluting 1 cc. of stock solution to 100 cc. Each cc. of the standard solution contained 0.0001 gm. of histidine dichloride.

The color develops slowly in this case. At first a yellow color is produced which changes rapidly to orange and then more slowly to pink. A maximum color value is obtained after 5 to 6 minutes, and is maintained in the weaker solutions for about 3 minutes. Then the color changes slowly through orange to yellow.

The previously described (CR-MO) indicator was used for comparisons. The color generated by histidine contains slightly more yellow than the standard indicator solution but the difference in shade is so slight, that with practice, an intensity comparison can be made with an accuracy of from 0.5 to 3 per cent, as has been demonstrated by the determination of unknowns. Where histidine is the only substance to be determined a more perfect color match can be obtained by using more of the stock methyl orange solution in compounding the standard indicator solution. If this is done, however, Table I cannot be used. We have preferred to use this standard indicator solution because, as will be shown in the following two articles, it is more universally applicable to mixtures of imidazoles than any other.

TABLE I.

Estimation of Small Amounts of Histidine.

Depth of indicator solution (CR-MO) required to match the color in the test cylinder.	Histidine dichloride in the test cylinder (total volume 8 cc.). Test cylinder set at 20 mm.
mm.	*gm.*
0.5	0.000001
1.0	0.000002
1.5	0.000003
2.0	0.000004
2.5	0.000005
3.0	0.000006
3.5	0.000007
4.0	0.000008
4.5	0.000009
5.0	0.000010
5.5	0.000011
6.0	0.000012
6.5	0.000013
7.0	0.000014
7.5	0.000015
8.0	0.000016
8.5	0.000017
9.0	0.000018
9.5	0.000019
10.0	0.000020
10.5	0.000021
11.0	0.000022
11.5	0.000023
12.0	0.000024
12.5	0.000025
13.0	0.000026
13.5	0.000027
14.0	0.000028
14.5	0.000029
15.0	0.000030
15.5	0.000031
16.0	0.000032
16.5	0.000033
17.0	0.000034
17.5	0.000035
18.0	0.000036
18.5	0.000037
19.0	0.000038
19.5	0.000039
20.0	0.000040

TABLE I—*Concluded.*

Depth of indicator solution (CR-MO) required to match the color in the test cylinder.	Histidine dichloride in the test cylinder (total volume 8 cc.). Test cylinder set at 20 mm.
mm.	*gm.*
20.5	0.000041
21.0	0.000042
21.5	0.000043
22.0	0.000044
22.5	0.000045
23.0	0.000046
23.5	0.000047
24.0	0.000048
24.5	0.000049
25.0	0.000050

Table I shows clearly that the color production is directly proportional to the quantity of histidine dichloride used. This fact can be conveniently represented by the formula $I \times 0.000002 = H$, where I is the depth of the indicator solution in mm. required to match the color in the test cylinder, and H is the number of gm. of histidine dichloride in the test cylinder. Thus if the indicator solution had to be set at 18.5 mm. to match the color in the test cylinder, I would equal 18.5 and H would be equal to $18.5 \times 0.000002 = 0.000037$ gm. of histidine dichloride in the test cylinder.

Estimation of Small Amounts of Histamine (β-Imidazolylethylamine).

A stock histamine solution was prepared by dissolving 2.1097 gm. of 94.8 per cent histamine dichloride in water and diluting to exactly 200 cc. A layer of toluene was added as a preservative. From this the standard solution was prepared by diluting 1 cc. of the stock solution to 100 cc. Each cc. of the standard solution contained 0.0001 gm. of histamine dichloride.

The color develops quite rapidly in this case and becomes pink almost immediately. Although most of the color develops within 1 minute, a maximum is reached only after 4 to 5 minutes. With dilute solutions—0.00002 gm. or less—the color of maximum intensity persists for about 2 minutes. With more concentrated solutions, the period of maximum intensity may be as short as 30 seconds.

TABLE II.

Estimation of Small Amounts of Histamine.

Depth of indicator solution (CR-MO) required to match the color in the test cylinder.	Histamine dichloride in the test cylinder (total volume 8 cc.). Test cylinder set at 20 mm.
mm.	*gm.*
0.8	0.000001
1.5	0.000002
2.2	0.000003
3.0	0.000004
3.7	0.000005
4.5	0.000006
5.2	0.000007
6.0	0.000008
6.7	0.000009
7.5	0.000010
8.2	0.000011
9.0	0.000012
9.7	0.000013
10.5	0.000014
11.2	0.000015
12.0	0.000016
12.7	0.000017
13.5	0.000018
14.2	0.000019
15.0	0.000020
15.7	0.000021
16.5	0.000022
17.2	0.000023
18.0	0.000024
18.7	0.000025
19.5	0.000026
20.2	0.000027
21.0	0.000028
21.7	0.000029
22.5	0.000030
23.2	0.000031
24.0	0.000032
24.7	0.000033
25.5	0.000034
26.2	0.000035
27.0	0.000036
27.7	0.000037
28.5	0.000038
29.2	0.000039
30.0	0.000040

TABLE II—*Concluded.*

Depth of indicator solution (CR-MO) required to match the color in the test cylinder.	Histamine dichloride in the test cylinder (total volume 8 cc.). Test cylinder set at 20 mm.
mm.	*gm.*
30.7	0.000041
31.5	0.000042
32.2	0.000043
33.0	0.000044
33.7	0.000045
34.5	0.000046
35.2	0.000047
36.0	0.000048
36.7	0.000049
37.5	0.000050

The previously described (CR-MO) indicator matches the color produced by histamine perfectly.

From Table II the facts can be represented by a formula, namely,

$$I \times 0.00001333 \ldots = H_s$$

where H_s is the number of gm. of histamine in the test cylinder and I retains the meaning assigned to it under Table I.

Estimation of Small Amounts of Imidazole Propionic Acid.

A stock solution was prepared by dissolving 2.0000 gm. of 100 per cent imidazole propionic acid in water, adding enough of a normal solution of hydrochloric acid to convert it into the hydrochloride, and finally diluting with water to 200 cc. A few drops of chloroform were added as a preservative. From this the standard solution was prepared by diluting 1 cc. of the stock solution to 100 cc. Each cc. of the standard solution contained 0.0001 gm. of imidazole propionic acid.

A large per cent of the color develops immediately and it is red from the beginning. An accurate comparison with the indicator can be made at the end of 1 minute. Thus it was found that for 0.00001 gm. of substance a color value of 13.0 mm. (CR) was obtained at the end of 1 minute and 15.3 mm. at the end of 10 minutes which is the time required to give a maximum color at 20°. The rate of development depends to a large extent upon the temperature. Thus at 18°, 13 minutes were required to give

TABLE III.

Estimation of Small Amounts of Imidazole Propionic Acid.

Depth of indicator solution (CR) required to match the color in the test cylinder.	Imidazole propionic acid in the test cylinder (total volume 8 cc.). Test cylinder set at 20 mm.
mm.	*gm.*
1.5	0.000001
3.0	0.000002
4.5	0.000003
6.0	0.000004
7.5	0.000005
9.0	0.000006
10.5	0.000007
12.0	0.000008
13.5	0.000009
15.0	0.000010
16.5	0.000011
18.0	0.000012
19.5	0.000013
21.0	0.000014
22.5	0.000015
24.0	0.000016
25.5	0.000017
27.0	0.000018
28.5	0.000019
30.0	0.000020
31.5	0.000021
33.0	0.000022
34.5	0.000023
36.0	0.000024
37.5	0.000025
39.0	0.000026
40.5	0.000027
42.0	0.000028
43.5	0.000029
45.0	0.000030

a maximum of color while at 22° only 8 minutes were required. The *amount* of color finally produced is always the same regardless of the temperature. The color is unusually stable. After reaching its maximum there is no apparent change for 5 minutes.

The previously described (CR) indicator matches the color produced by imidazole propionic acid perfectly.

Table III was not extended because the colors were too intense to make an accurate comparison possible. The values higher than 25 mm. of indicator solution were obtained by setting the test cylinder at 10 instead of 20 mm. and multiplying the reading obtained by 2. The facts can in this case be represented by the formula $I \times 0.0000006666 \ldots = P$ where P is the number of gm. of imidazole propionic acid in the test cylinder.

Estimation of Small Amounts of Imidazole Acetic Acid.

A stock solution was prepared by dissolving 2.0000 gm. of 100 per cent imidazole acetic acid hydrochloride in water and diluting to exactly 200 cc. A few drops of chloroform were added as a preservative. From this the standard solution was prepared by diluting 1 cc. of the stock solution to 100 cc. Each cc. of the standard solution contained 0.0001 gm. of the hydrochloride of imidazole acetic acid.

The (CR) indicator matches the color produced by imidazole acetic acid perfectly. Table IV shows the results of the test.

The statements given above for imidazole propionic acid apply equally well in this case. The facts can be represented by the formula $I \times 0.00000081308 = A$, where A is the number of gm. of imidazole acetic acid in the test cylinder.

Estimation of Small Amounts of Methyl Imidazole.

A stock solution was prepared by dissolving 2.0000 gm. of the base in 24.4 cc. of N HCl and diluting with water to exactly 200 cc. A few drops of chloroform were added as a preservative. From this the standard solution was prepared by diluting 1 cc. of the stock solution to 100 cc. Each cc. of the standard solution contained 0.0001 gm. of methyl imidazole.

A large per cent of the color develops immediately which is red and comparable with the indicator from the start. The color reaches its maximum intensity in 7 minutes at 20°, and undergoes no apparent change for 15 minutes. The (CR) indicator was used for comparisons. The color match is not quite perfect in this case, as the color produced by the methyl imidazole is slightly too red for the (CR) indicator. The discrepancy in shade is so slight, however, that an accurate comparison can easily be made.

TABLE IV.

Estimation of Small Amounts of Imidazole Acetic Acid.

Depth of indicator solution (CR) required to match the color in the test cylinder.	Imidazole acetic acid hydrochloride in the test cylinder (total volume 8 cc.). Test cylinder set at 20 mm.
mm.	*gm.*
1.2	0.000001
2.5	0.000002
3.7	0.000003
4.9	0.000004
6.1	0.000005
7.4	0.000006
8.6	0.000007
9.8	0.000008
11.1	0.000009
12.3	0.000010
13.5	0.000011
14.7	0.000012
16.0	0.000013
17.2	0.000014
18.5	0.000015
19.7	0.000016
20.9	0.000017
22.1	0.000018
23.4	0.000019
24.6	0.000020
25.8	0.000021
27.0	0.000022
28.3	0.000023
29.5	0.000024
30.7	0.000025
32.0	0.000026
33.2	0.000027
34.4	0.000028
35.7	0.000029
36.9	0.000030
38.1	0.000031
39.4	0.000032
40.6	0.000033
41.9	0.000034
43.0	0.000035
44.3	0.000036
45.5	0.000037
46.7	0.000038
47.9	0.000039
49.2	0.000040

Table V was not extended beyond 0.00002 gm. of methyl imidazole because the colors were too intense to make an accurate comparison possible. The values higher than 25 mm. of indicator solution were obtained by setting the test cylinder at 10 instead of 20 mm. and multiplying the reading obtained by 2. The facts can be represented by the formula $I \times 0.00000037037 \ldots = M$ where M is the number of gm. of methyl imidazole in the test cylinder.

TABLE V.

Estimation of Small Amounts of Methyl Imidazole.

Depth of indicator solution (CR) required to match the color in the test cylinder.	Methyl imidazole in the test cylinder (total volume 8 cc.). Test cylinder set at 20 mm.
mm.	*gm.*
2.7	0.000001
5.4	0.000002
8.1	0.000003
10.8	0.000004
13.5	0.000005
16.2	0.000006
18.9	0.000007
21.6	0.000008
24.3	0.000009
27.0	0.000010
29.7	0.000011
32.4	0.000012
35.1	0.000013
37.8	0.000014
40.5	0.000015
43.2	0.000016
45.9	0.000017
48.6	0.000018
51.3	0.000019
54.0	0.000020

Relative Value of the Standard Indicator Solutions.

The relative intensity of the two standard indicator solutions can be compared by means of the Duboscq colorimeter even though the tint is not identical. This enables one to compare readings obtained with the (CR) indicator with those obtained with the (CR-MO) indicator. The readings obtained, representing mm. on the Duboscq scale, are as follows.

(CR) standard indicator.	(CR-MO) standard indicator.
11	10
22	20
33	30

The following example will illustrate the use of the table. 0.00001 gm. of the hydrochloride of glyoxaline acetic acid has a color value on the (CR) scale of 12.3 mm. by actual determination. A calculation from the table shows that the color value on the (CR-MO) scale should be $\frac{10}{11} \times 12.3 = 11.2$ mm. The actual determination using the (CR-MO) scale, which was made fully 2 months before this comparison table was compiled, actually gave 11.2 mm. It is now possible to compare the molecular color values of the various imidazoles.

Molecular Color Value of the Five Imidazoles.

The following figures were obtained by calculating the hypothetical color value on the (CR-MO) scale that would be obtained if one molecular weight of the imidazole had been present in the test cylinder. The calculations are based on the values recorded in the tables for 0.00001 gm. of the imidazole derivatives.

	Weight. *mol*	Molecular color value.
Histidine dichloride	228.0	114,000,000
Histamine "	184.0	138,000,000
Imidazole propionic acid	140.0	191,000,000
" acetic acid as HCl	162.5	182,000,000
Methyl Imidazole	82.0	201,000,000

From this tabulation the following conclusions can be drawn.

1. The color produced by these five imidazoles depends not only upon the imidazole ring but is strongly influenced by the groups in the side chain also.

2. The color is nearly a pure red and of maximum intensity when the side chain is paraffin in character (methyl imidazole).

3. The carboxyl group makes the color more yellow and reduces its intensity. This effect is most marked when the carboxyl group is near the imidazole ring. Thus imidazole acetic acid has a molecular color value that is about 90 per cent as great as that of methyl imidazole, whereas imidazole propionic acid has a molecular color value that is 95 per cent as great as that of methyl imidazole.

4. The introduction of an amino group greatly reduces the intensity of the color and makes it decidedly yellow. When both the amino and the carboxyl group are present in the same molecule, the effect on the color is additive. Thus histamine has a molecular color value that is about 69 per cent as great as that of the methyl imidazole whereas histidine has a molecular color value that is only 57 per cent as great as that of methyl imidazole.

The Correction Blank.

When the reagent and alkali are mixed in the absence of an imidazole derivative, a very pale yellow color is produced in the course of 5 to 10 minutes that has an intensity value of 0.30 mm. (CR-MO). This same quantity of color is also produced in the presence of imidazole derivatives along with the color produced by the imidazole; so the readings obtained are too high by 0.30 mm. in every case. It is necessary, therefore, to subtract 0.30 mm. from the readings obtained before comparison with the tables. For example, 0.10 cc. of a histidine solution is found to have a color value equivalent to 10.3 mm. (CR-MO). The corrected reading is 10.0 mm. (CR-MO); so 0.10 cc. of this solution contains 0.00002 gm. of histidine dichloride (Table I). It is hardly necessary to say that the correction blank will be 0.30 mm. only when the test cylinder is set at 20 mm.

Substances That Do and Do Not Interfere with the Quantitative Determination of Histidine.

2 cc. of stock histidine solution were mixed in a 100 cc. precision cylinder with quantities of the other substances as stated in Table VI. Water was then added to give a final volume of 100 cc. Then 0.10 cc. of the solution was taken for the colorimetric determination.

The most active interfering substances are apparently ketones, alcohols, and ammonium salts. The volatile ketones and alcohols can always be removed by evaporation; so they offer no permanent difficulties. Amyl alcohol can be completely removed from an aqueous solution by extraction with toluene or chloroform. The resulting aqueous solution gives the correct color value. The

dissolved chloroform or toluene does not interfere with the production of color. Glycerol or glucose is seldom present, in concentrations as high as those given here, in the usual biological liquids.

TABLE VI.

Substance.	Concentration	Color.	Interference.
	per cent		
Urea.	5	Perfect.	None.
NaCl KCl Na_2SO_4 KH_2PO_4	5	"	"
Sodium acetate. Sodium citrate.	5	"	"
Glycerol.	3	"	2 per cent low in color value.
Glucose.	5	Slightly too yellow.	8 " " " " " "
Ethyl alcohol.	5 10	Perfect.	5 " " " " " " 10 " " " " " "
Methyl alcohol.	10	Slightly too yellow.	20 " " " " " "
Amyl alcohol.	Saturated aqueous solution.	" "	10 " " " " " "
Acetone.	10	Greenish yellow.	Entirely unmatchable color of slight intensity. No determination possible.
$(NH_4)_2SO_4$ free from pyridine.	1 5	Slightly brown. Greenish "	2 per cent high in color value. Entirely unmatchable color of high intensity. No determination possible.

The following substances require individual consideration.

Ammonium salts.—The positive interference referred to above for ammonium sulfate led us to believe that ammonia itself gave

a color with the reagent. Experiment proved this to be the case. A 10 per cent aqueous solution of the highest purity of ammonium sulfate obtainable, which was free from pyridine, was tested for color value by introducing 0.10 cc. of it into the test cylinder in the usual manner. A fairly intense yellowish green color developed promptly. Exactly the same quantity and quality of color were obtained when 0.50 cc. of the ammonium sulfate solution was tested.

The color value of a solution containing more than the equivalent of 1 per cent of ammonium sulfate is untrustworthy.

Proteins.—The colorimetric method is worthless when applied to a solution containing soluble proteins for two reasons. (*a*) The proteins themselves give a color with the reagent which is not quantitatively produced. Thus 0.20 cc. of a protein solution gives less than twice the color produced by 0.10 cc. (*b*) The addition of a known amount of histidine to a solution containing protein of previously determined color value gives less than the theoretical increase in color value. The results with blood proteins run about 30 per cent low.

It might be possible to eliminate this protein interference by using a stronger reagent. This would make it necessary to develop a new set of tables. No experiments have been made up to the present time to verify the correctness of the above statement.

Animal Charcoal.—We have found that animal charcoal cannot be used to decolorize a solution in which the presence of small amounts of imidazoles is to be determined, because the charcoal adsorbs appreciable quantities of these substances. Experimental data are given in Table VII to support the above statement. Kahlbaum's highest grade of powdered bone charcoal was used in these experiments. A weighed quantity of the charcoal was agitated for 10 minutes with 100 cc. of the imidazole solution. The color value of the clear solution was then determined.

Table VII shows clearly that for preparative work, or even for quantitative work where the concentration of the imidazole is high, charcoal might be used with impunity because the maximum adsorption per gm. of charcoal seems to be about 1 mg. of imidazole. It is best, however, to avoid the use of charcoal when possible.

Barium sulfate precipitates do not adsorb imidazoles.

The general method outlined will undoubtedly be found to be equally applicable to the estimation of all imidazoles with the possible exception of cyanomethyl imidazole which gives a brown and not an orange or red color. It is also applicable to the estimation of other compounds that will couple with *p*-diazobenzene sulfonic acid such as phenols and it may prove useful for the

TABLE VII.

Charcoal added.	Amount and kind of imidazole used (total volume 100 cc.).	Color value of indicator solution before adding charcoal.	Color value of indicator solution after adding charcoal.	Imidazole adsorbed by the charcoal.	
gm.	*gm.*	*mm.*	*mm.*	*gm.*	*per cent*
1	0.01 methyl imidazole.	0.10 cc. equivalent to 27.3 mm. (CR).	0.10 cc. equivalent to 9.5 mm. (CR).	0.0066	66
2	0.01 methyl imidazole.	0.10 cc. equivalent to 27.3 mm. (CR).	0.10 cc. equivalent to 5.5 mm. (CR).	0.0080	80
4	0.01 methyl imidazole.	0.10 cc. equivalent to 27.3 mm. (CR).	0.10 cc. equivalent to 3.0 mm. (CR).	0.0090	90
1	0.02 methyl imidazole.	0.10 cc. equivalent to 54.3 mm. (CR).	0.10 cc. equivalent to 33.5 mm. (CR).	0.0075	37.5
1	0.01 histamine dichloride.	0.20 cc. equivalent to 15.3 mm. (CR-MO).	0.20 cc. equivalent to 2.0 mm. (CR-MO).	0.0090	90

estimation of aromatic amines. We hope to report the method for estimating phenols in the near future.

Our interest in these particular imidazoles will be made clear in the succeeding two papers.

SUMMARY.

1. The well known interaction between the imidazole ring and *p*-phenyldiazonium sulfonate has been made the basis of a quantitative colorimetric method for estimating histidine, histamine,

imidazole propionic acid, imidazole acetic acid, and methyl imidazole.

2. Tables are given for the direct determination of quantities of these substances ranging from 0.000001 to 0.00005 gm. The amount of imidazole in any quantity of liquid can then be determined, by multiplication, with an accuracy of from 0.5 to 3 per cent.

3. A list of substances is given, some of which do and others of which do not interfere with the determination.

STUDIES ON PROTEINOGENOUS AMINES.

III. A QUANTITATIVE METHOD FOR SEPARATING HISTAMINE FROM HISTIDINE.

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An exact study of the metabolism of histidine has been very difficult up to the present time because we do not have the proper quantitative method for estimating imidazole derivatives. It has always been necesssary, therefore, either to isolate the imidazole derivative or to determine its presence by biological reactions. The isolation of these compounds from the usual body liquids is a very difficult task and the quantity actually isolated is probably never more than a small per cent of that actually present. The large amounts of material necessary for the isolation of imidazoles present in small quantities make the process of isolation so tedious and time-consuming that it is hardly applicable to cases where a routine analysis is desired. The biological methods, based on the action of histamine on the smooth muscle fiber system, that have been used for the identification of the base, are certainly not quantitatively exact and have not been shown to be entirely specific. A purely chemical method, applicable to small amounts of material, would be superior to any that had been previously described, if the new method were accurate and practically applicable.

In the preceding communication a method for estimating small amounts of imidazoles has been described. In that paper no claims were made for the applicability of the method to mixtures of imidazoles. It is the object of this report to show that the method can also be applied to mixtures and that the exact quantity of histamine, histidine, and methyl imidazole in such mixtures can be rapidly and accurately determined. The method has been

found to be applicable without modification to liquid media in which bacteria have been allowed to metabolize histidine in the presence of salts and glycerol or glucose.[1] In its present form the method is not directly applicable, in its entirety, to more complex liquids such as urine or blood. We are trying to modify it, at the present time, so that it can be applied to such liquids. The underlying principles of the method are as follows.

The mixture of imidazole derivatives is treated with enough solid sodium hydroxide to give a 20 per cent solution of the alkali. This alkaline liquid is then extracted six times with twice its volume of amyl alcohol. This effects a separation into two fractions, the amyl alcohol extract and the alkaline aqueous liquid. The amyl alcohol extract contains all the histamine and methyl imidazole, together with ammonia and other amines if such are present in the original solution. We shall call this the *histamine fraction.* The alkaline aqueous liquid contains all the histidine, imidazole propionic, acetic, and lactic acids. We shall call this the *histidine fraction.*

The amount of histidine can then be determined by an amino nitrogen determination on the histidine fraction.

A color value determination on the histidine fraction is an index of the combined imidazole content of the liquid. If the color value determination indicates the presence of more imidazole than can be accounted for on the basis of the amino nitrogen determination, histidine is not the only imidazole present. The excess of color value can then be calculated to imidazole propionic, acetic, or lactic acids depending upon which of these substances is shown to be present by actual isolation.

Of all the imidazoles tried, methyl imidazole is the only one that is volatile with steam. It can be determined directly by collecting the distillates from a steam distillation, determining their color value, and calculating the amount of methyl imidazole from Table V of the previous article. It may be well to call attention here to the fact that imidazole itself is probably also volatile with, steam and if it is present, it will be estimated as methyl imidazole. No effort has been made to differentiate these two substances.

[1] Koessler, K. K., and Hanke, M. T., *J. Biol. Chem.*, 1919, xxxix, 539.

The histamine and methyl imidazole can be completely extracted from the amyl alcohol with 1.0 N H_2SO_4. A color value determination on the acid extract gives the total amount of imidazole present. By subtracting from this the color value due to the methyl imidazole, as previously determined, the color value due to histamine is obtained which can then be calculated to that substance by using Table II given in the previous paper. The bacterial experiments, described in the succeeding paper, showed methyl imidazole to be regularly absent. In this case, then, the color value determination gave the amount of histamine directly.

Sections I to VI of this report contain a detailed account of the results of experiments on known solutions of imidazole derivatives by which the accuracy of the technique of the method for separating histamine from histidine, described in Section VII, was experimentally established.

EXPERIMENTAL.[2]

I. Color Value of a Histidine-Histamine Mixture is the Sum of Their Individual Color Values.

(*a*) 1 cc. each of stock 1 per cent histidine and histamine dichloride solutions was mixed in a volumetric flask and diluted with water to exactly 100 cc.

Of this solution 0.10 cc. had a color value equivalent to 12.6 mm. (CR-MO).

This amount of each of the imidazoles alone would give

Histidine	5.0 mm.	(CR-MO)
Histamine	7.5 "	"
Theory	12.5 "	"

(*b*) 1 cc. of the stock histamine solution and 0.50 cc. of the stock histidine solution were mixed in a volumetric flask and diluted with water to exactly 100 cc.

Of this solution 0.10 cc. had a color value equivalent to 10.0 mm. (CR-MO).

[2] The reagents and the terminology used are identical with those of the previous article (p. 497). The recorded readings have all been corrected by subtracting 0.30 mm., for the correction blank (p. 511).

This amount of each of the imidazoles alone would give

Histidine	2.5 mm.	(CR-MO)
Histamine	7.5 "	"
Theory	10.0 "	"

Conclusion.—Histidine and histamine do not interfere with each other's color production. Each produces its color as if the other were absent.

II. Stability of Histidine and Histamine toward Acid and Alkali.

(*a*) *Cold 20 Per Cent Sodium Hydroxide.*—1 cc. each of the stock histidine and histamine solutions was mixed with 2 gm. of solid sodium hydroxide and enough water to give a total volume of 10 cc. After standing for 1 hour at room temperature the liquid was rendered faintly acid to litmus paper with 1.0 N HCl and diluted to exactly 100 cc.

Of this solution 0.10 cc. had a color value equivalent to 12.7 mm. (CR-MO).

The theory is 12.5 mm. (CR-MO) (see Section I) the result, therefore, is accurate within the limits of experimental error.

(*b*) *Hot 20 Per Cent Sodium Hydroxide.*—2 cc. of stock histidine solution were mixed with 8 cc. of water and 2 gm. of solid sodium hydroxide in a 100 cc. volumetric flask. The flask was heated on the boiling water bath for 7 hours. The cooled solution was neutralized as in (*a*) and diluted to 100 cc.

Of this solution 0.10 cc. had a color value equivalent to 8.4 mm. (CR-MO).

This, by table, is equal to 84 per cent of the histidine started with. The 7 hours heating with concentrated sodium hydroxide destroyed the imidazole ring to the extent of 16 per cent.

(*c*) An experiment was conducted on 2 cc. of stock histamine solution, the details of which were identical with those described under (*b*).

Of the final solution 0.10 cc. had a color value equivalent to 13.8 mm. (CR-MO).

This, by table, is equal to 92.5 per cent of the histamine started with. The 7 hours heating with concentrated alkali destroyed the imidazole ring to the extent of 7.5 per cent.

(*d*) *Hot Concentrated Hydrochloric Acid.*—2.00 cc. of stock histidine solution and 25 cc. of 37 per cent HCl were mixed in a 100 cc. lipped beaker and heated for 10 hours on the boiling water bath, rapid evaporation being prevented by covering the beaker with a watch-glass. At the end of the 10 hours heating the hydrochloric acid had disappeared. The dry residue was dissolved in water and diluted to exactly 100 cc.

Of this solution 0.10 cc. had a color value equivalent to 10.0 mm. (CR-MO).

This is the theoretical value.

(*e*) An experiment was conducted on 2 cc. of stock histamine solution, the details of which were identical with those described under (*d*).

0.10 cc. of the final solution had a color value equivalent to 15.0 mm. (CR-MO).

This is the theoretical value.

Conclusions.—Cold 20 per cent sodium hydroxide and hot concentrated hydrochloric acid do not change the color value of either histidine or histamine. Hot 20 per cent sodium hydroxide destroys 7.5 and 16 per cent of the color values of histamine and histidine respectively in the course of 7 hours. Since this loss in color value is probably caused by a destruction of the imidazole ring, it is clear that this heterocyclic ring is not perfectly stable toward hot alkali. The stability of the ring seems to depend to some extent upon the character of the side chain.

For quantitative results, a solution containing imidazoles may be evaporated to dryness with hydrochloric acid with impunity. Heating a strongly alkaline solution must be avoided.

III. Histidine Not Extracted from a 20 Per Cent Sodium Hydroxide Solution by Amyl Alcohol.

In order to prove that histidine is not extracted from a 20 per cent sodium hydroxide solution by amyl alcohol it was necessary to devise a method for extracting the alkaline solution with amyl alcohol that would be rapid and accurate. The following technique was finally adopted.

Extraction Tube and Mode of Extraction.—A 35 cc. glass-stoppered bottle was used as an extraction tube. The imidazole so-

lution, in this case 1.00 cc. of stock histidine solution, was introduced into the bottle together with enough water to give a total volume of about 10 cc. Then 2 gm. of Merck's highest purity sodium hydroxide were dissolved in the liquid, the bottle and con-

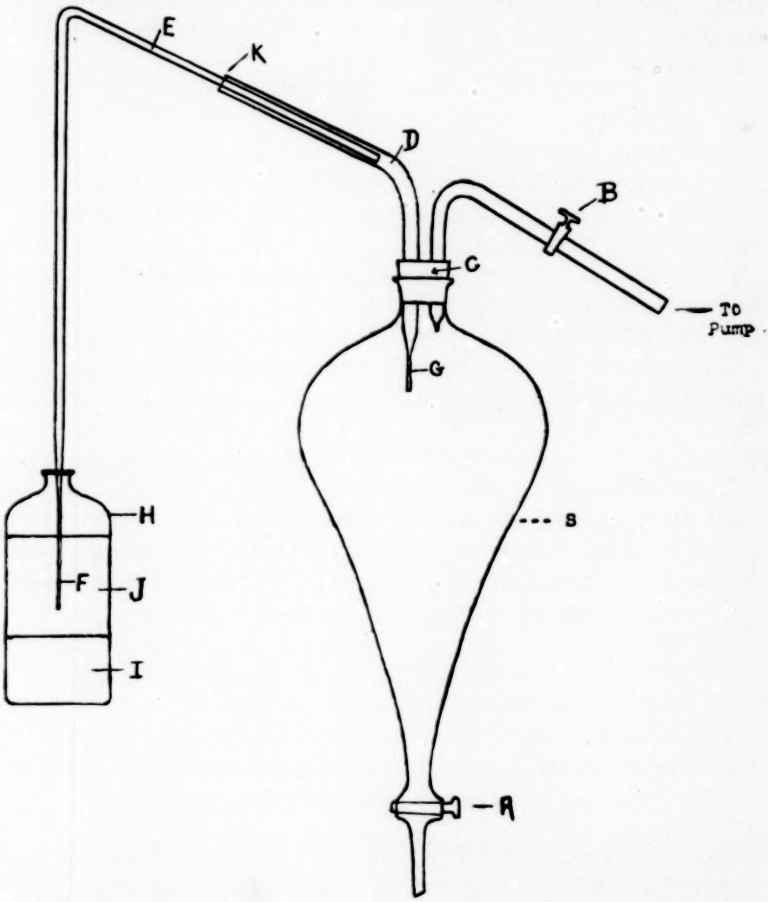

FIG. 1. A, Lower stop-cock of the Squibb separatory funnel S. B, Stop-cock to outlet tube. C, Two-hole rubber stopper. D, Wide glass tube. E, Narrow glass tube. F, Capillary tube. G, Constriction in D, about 1 mm. diameter. H, Glass-stoppered extraction tube. I, Aqueous layer. J, Amyl alcohol layer. K, A piece of rubber tubing is used at this place to connect Tubes D and E.

tents being cooled in tap water while the alkali was dissolving. 20 cc. of prepared amyl alcohol,[3] measured by graduate, were introduced into the bottle, the glass stopper[4] inserted, and the liquids vigorously mixed for a few minutes. The bottle was then transferred to a centrifuge tube[5] and centrifuged for from 1 to 2 minutes. This gave a sharp separation into two layers.

Separation of Amyl Alcohol.—The amyl alcohol layer was separated from the aqueous alkaline layer by means of the device shown in Fig. 1.

The 300 cc. Squibb funnel S, which must be equipped with a tightly fitting glass stopper, and the extraction tube H are supported by means of two universal clamps which in turn are attached to the same ring-stand.

After the mixture in the extraction tube has been centrifuged into two sharply defined layers, the tube is clamped into place and raised until the capillary F is immersed in the amyl alcohol layer. Stop-cock B, which should be attached to an aspirator, is then opened very slightly so that a very slight drop in pressure occurs in the Squibb funnel. The amyl alcohol is sucked through tubes E and D into the funnel. By carefully raising the extraction tube H, the amyl alcohol can be almost entirely removed without disturbing the lower layer I. The extraction tube H is then lowered, tube E swung around so that capillary F points straight up and stop-cock B opened wide to sweep the amyl alcohol from tubes D and E into the funnel.

The alkaline aqueous layer is then ready to be extracted with another 20 cc. portion of amyl alcohol. The above process was repeated five times in this case, so that a total of 100 cc. of amyl alcohol was used.

[3] The amyl alcohol was prepared by distilling Merck's highest purity product *in vacuo* and extracting the distillate once with 20 per cent sodium hydroxide solution. This gives a product that is already saturated with water and sodium hydroxide with respect to a 20 per cent sodium hydroxide solution. The volume of the aqueous alkaline layer in the extraction tube will, therefore, remain constant.

[4] The glass stopper must fit perfectly throughout its entire length. It must not be removed from the bottle until after the liquid has been centrifuged.

[5] A deep layer of cotton makes an ideal cushion for the flat bottomed bottle.

Removal of Imidazoles from Amyl Alcohol.—The combined amyl alcohol extracts were extracted five times in the same Squibb funnel with 1.0 N H_2SO_4, using 20 cc. for the first and 10 cc. for each of the remaining four extracts. The sulfuric acid extracts were collected in a 100 cc. glass-stoppered precision cylinder and neutralized to litmus paper with 40 per cent sodium hydroxide. The solution was then rendered very faintly acid by adding a few drops of 1.0 N H_2SO_4, cooled to room temperature in tap water, and diluted to exactly 100 cc. with distilled water. About 20 cc. of chloroform were then added[6] and the mixture was vigorously agitated. The clear aqueous layer was used for the colorimetric determinations.

In this case 1.0 cc. had a color value equivalent to 0.20 mm. (CR-MO) which, by table, is equal to about 0.40 per cent of the histidine originally introduced.

The Alkaline Aqueous Liquid (Histidine Fraction).—The alkaline aqueous liquid was carefully transferred with water from the extraction tube to a 100 cc. precision cylinder, made slightly acid with hydrochloric acid, and, after cooling to room temperature, diluted to 100 cc. About 20 cc. of chloroform were then added[6] and the mixture was vigorously agitated. The clear aqueous layer was used for the colorimetric determinations.

In this case 0.10 cc. had a color value equivalent to 5.0 mm. (CR-MO); 0.20 cc. had a color value equivalent to 10.0 mm. (CR-MO), which, by table, is equivalent to 100 per cent of the histidine introduced.

Conclusion.—Histidine is extracted from a 20 per cent sodium hydroxide solution only to the extent of about 0.50 per cent by amyl alcohol under the prescribed conditions. This is negligible in most cases.

IV. Histamine Quantitatively Extracted from a 20 Per Cent Sodium Hydroxide Solution by Amyl Alcohol.

1 cc. of stock 1 per cent histamine solution was diluted to 10 cc. in the extraction tube. 2 gm. of solid sodium hydroxide were

[6] The chloroform removes the amyl alcohol from the aqueous layer completely. Amyl alcohol interferes actively with the colorimetric determinations; so it is necessary to take this precaution to be sure that the readings obtained are accurate.

then added and the alkaline aqueous liquid extracted six times with prepared amyl alcohol. The details of this experiment were duplicates of those described under Section III.

Alkaline Aqueous Liquid.—The solution finally obtained had absolutely no color value beyond that of the correction blank.

Amyl Alcohol Extract (Histamine Fraction).—Of the solution finally obtained, 0.10 cc. had a color value equivalent to 7.5 mm. (CR-MO); 0.20 cc. had a color value equivalent to 15.0 mm. (CR-MO). This, by table, is equivalent to 100 per cent of the histamine started with.

Conclusion.—Histamine is quantitatively extracted from a 20 per cent sodium hydroxide solution by amyl alcohol.

V. Histamine Quantitatively Separated from Histidine by Means of Amyl Alcohol.

(A) When Equal Parts by Weight of Histidine and Histamine Are Present.

1 cc. each of stock histidine and histamine solutions was diluted to 10 cc. in the extraction tube. 2 gm. of solid sodium hydroxide were then added and the alkaline aqueous liquid extracted five times with prepared amyl alcohol. The details of this experiment were duplicates of those described under Section III.

Alkaline Aqueous Liquid (Histidine Fraction).—Of the solution finally obtained, 0.20 cc. had a color value equivalent to 10.2 mm. (CR-MO). This is equivalent to 0.0102 gm. of histidine dichloride which is 102 per cent of the histidine originally introduced.

Amyl Alcohol Extract (Histamine Fraction).—Of the solution finally obtained, 0.10 cc. had a color value equivalent to 7.4 mm. (CR-MO); 0.20 cc. had a color value equivalent to 14.7 mm. (CR-MO). This is equivalent to 0.0098 gm. of histamine dichloride which is 98 per cent of the starting material.

Discussion.—In this case both results are accurate to within the limit of experimental error; but we are now certain that the low value for the histamine fraction was obtained because too few amyl alcohol extracts were made. In the following two experiments where six extracts were made instead of five, the agreement

was perfect. It is necessary, therefore, to make not less than six amyl alcohol extracts to effect a quantitative separation of histamine from histidine.

(B) When a Small Amount of Histamine Is Mixed with a Large Amount of Histidine.

5 cc. of stock histidine solution and 0.30 cc. of stock histamine solution were diluted to 10 cc. in the extraction tube. 2 gm. of solid sodium hydroxide were then added and the alkaline aqueous liquid extracted six times with prepared amyl alcohol. The details of this experiment were duplicates of those described under Section III.

Histidine Fraction.—As the solution finally obtained was too concentrated to make an accurate determination possible 10 cc. of it were diluted to 100 cc. Of this diluted solution, 0.40 cc. had a color value equivalent to 10.0 mm. (CR-MO). This is equivalent to 0.050 gm. of histidine dichloride for the entire original solution which is a 100 per cent recovery.

Histamine Fraction.—Of the solution finally obtained, 0.10 cc. had a color value equivalent to 22.7 mm. (CR-MO). This is equivalent to 0.003025 gm. of histamine dichloride for the entire solution which is 100.9 per cent of the starting material.

(C) When a Large Amount of Histamine is Mixed with a Small Amount of Histidine.

4.5 cc. of stock histamine solution and 0.50 cc. of stock histidine solution were diluted to 10 cc. in the extraction tube. 2 gm. of solid sodium hydroxide were then added and the alkaline aqueous liquid extracted six times with prepared amyl alcohol. The details of this experiment were duplicates of those described under Section III.

Histidine Fraction.—Of the solution finally obtained, 0.40 cc. had a color value equivalent to 10.0 mm. (CR-MO). This is equivalent to 0.005 gm. of histidine dichloride for the entire solution which is 100 per cent of the amount originally introduced.

Histamine Fraction.—As the solution finally obtained was too concentrated to make an accurate determination possible 10 cc. of it were diluted to 100 cc.

Of this diluted solution 0.40 cc. had a color value equivalent to 13.5 mm. (CR-MO). This is equivalent to 0.045 gm. of histamine dichloride for the entire solution which is 100 per cent of the amount originally introduced.

Conclusion.—Histamine can be quantitatively separated from histidine regardless of the proportion in which each is present.

VI. Estimation of Methyl Imidazole.

(A) Methyl Imidazole Slightly Volatile with Steam from Alkaline Solution[7] *(Method A).*

1 cc. of stock 1 per cent methyl imidazole was diluted to 100 cc. in a glass dish, treated with 5 cc. of a M Na_2CO_3 solution, and evaporated on the boiling water bath until the final volume of the alkaline liquid was about 5 cc. An excess of hydrochloric acid was then carefully added and the solution evaporated until all the water and acid had volatilized. The residue was transferred, with water, to a 100 cc. volumetric flask and diluted to exactly 100 cc.

Of this solution 0.10 cc. had a color value equivalent to 20.2 mm. (CR), which is equivalent to 0.0075 gm. of methyl imidazole or 75 per cent of the amount originally introduced. This treatment will volatilize 0.0025 gm. of methyl imidazole; so if less than this amount is present in the solution it should be determinable by this method which we shall call Method A. The mode of applying this method to a practical unknown is given in Section VII.

(B) Methyl Imidazole Volatilizes Slightly from a Solution Containing Hydrochloric Acid.[7]

1 cc. of stock methyl imidazole was diluted with water to about 100 cc. in a glass dish. Then 5 cc. of 37 per cent HCl were added and the solution evaporated on the water bath until all the water and acid had been volatilized. The residue was transferred to a volumetric flask and diluted with water to 100 cc.

[7] These two experiments, (*A*) and (*B*), do not prove that the methyl imidazole was actually lost by volatilization. That this imidazole derivative is really volatile with steam was conclusively demonstrated and the proof of this statement is given under Method B.

Of the solution so obtained 0.10 cc. had a color value equivalent to 23.7 mm. (CR), which is equivalent to 0.00875 gm. of methyl imidazole or 87.5 per cent of the amount originally introduced. It appears, then, that 0.00125 gm. of methyl imidazole volatilized even in the presence of hydrochloric acid. This may mean either that methyl imidazole hydrochloride is hydrolyzed by water and that the base formed by hydrolysis is volatilized, or that methyl imidazole hydrochloride is itself volatile. The following experiment has convinced us that it is the hydrochloride that is volatile.

These experiments are of value because they show clearly that a solution in which methyl imidazole is to be determined must not have been evaporated in the presence of either alkali or hydrochloric acid.

(C) Methyl Imidazole Will Not Volatilize from a Solution Containing Sulfuric Acid.

1 cc. of stock methyl imidazole solution was diluted with 100 cc. of water in a glass dish. Then 1 cc. of 95 per cent H_2SO_4 was added to the solution and the liquid evaporated to a final volume of about 5 cc. This was transferred to a volumetric flask, neutralized to litmus paper, and diluted to 100 cc.

Of this solution 0.10 cc. had a color value equivalent to 27.0 mm. (CR), which is equivalent to 100 per cent of the amount introduced.

When it is necessary to concentrate a solution before determining its color value, this can be safely done after adding an excess of sulfuric acid.

(D) Direct Determination of Methyl Imidazole (Method B).

5 gm. of anhydrous sodium carbonate, 5 cc. of water, and 1 cc. of stock methyl imidazole solution were subjected to a steam distillation from a 200 cc. distilling flask, the volume of the methyl imidazole-containing solution being kept constantly at from 5 to 10 cc.[8] The distillate was collected in a 250 cc. graduated precision cylinder.

[8] The liquid from which the methyl imidazole is to be volatilized must be kept saturated with sodium carbonate. Methyl imidazole is practically non-volatile from a dilute sodium carbonate solution.

Collections.—The volume of the first distillate was 154 cc., of which 0.10 cc. had a color value equivalent to 14.8 mm. (CR). The entire 154 cc. contained 0.00847 gm. of methyl imidazole.

The volume of the second distillate was 153 cc. of which 0.50 cc. had a color value equivalent to 8.7 mm. (CR). The entire 153 cc. contained 0.0009945 gm. of methyl imidazole.

The volume of the third distillate was 150 cc. of which 1.0 cc. had a color value equivalent to 6.0 mm. (CR). The entire 150 cc. contained 0.000338 gm. of methyl imidazole.

The volume of the fourth distillate was 140 cc. of which 1.0 cc. had a color value equivalent to 1.10 mm. (CR). The entire 140 cc. contained 0.00007 gm. of methyl imidazole.

There was thus accounted for 0.0098725 gm. of methyl imidazole which is 98.7 per cent of the amount originally introduced.

(*E*) *Proof That Methyl Imidazole Can Be Estimated by Method B in the Presence of Other Imidazoles.*

1 cc. each of the stock histidine, histamine, methyl imidazole, imidazole propionic acid, and imidazole acetic acid solutions was mixed and subjected to steam distillation under conditions identical with those given above. Six distillates were collected, the combined content of which was 0.009831 gm. of methyl imidazole which is 98.31 per cent of the methyl imidazole originally introduced. This proves conclusively that of the five imidazoles tried, only methyl imidazole is volatile with steam, and that it can be separated from other imidazoles by this method. Since methyl imidazole is chemically similar to *p*-cresol and scatole, both of which have been found in animal excretions, it will be of interest to see if methyl imidazole cannot also be found in such excretions. We have been unable to establish this as a fact up to the present time.

(*F*) *Method C for Estimating Methyl Imidazole.*

Method A has proved to be untrustworthy when applied to solutions containing the bacterial decomposition products of glycerol because evaporation with alkali gives rise to non-volatile substances that interfere with the colorimetric determinations. The amyl alcohol extract from such solutions is free from these decomposition

products. It should be possible, therefore, to determine methyl imidazole by evaporating the *histamine fraction* with alkali as described under Method A, instead of the *original solution*. In this case a color value determination on the histamine fraction would give the total amount of imidazole present. The solution would then be evaporated to dryness with 5 cc. of M Na_2CO_3 solution, the residue dissolved in hydrochloric acid and the solution reevaporated. The residue would be dissolved in water and diluted to the original volume of the histamine fraction. The color value so obtained would be *lower* than that of the original histamine fraction only when methyl imidazole was originally present and the decrease in color value could easily be calculated to methyl imidazole. For bacterial metabolism studies this method has been found to be quite accurate and shorter than Method B. Its application is more specifically given in Section VII.

VII. Separation of Histamine and Methyl Imidazole from Histidine, Imidazole Propionic Acid, and Imidazole Acetic Acid. The Accurate Determination of Histamine, Methyl Imidazole, and Histidine.

The method outlined below is a duplicate of the method that was used in the bacterial metabolism studies to be described in the following paper. Some of the steps were taken, not because they were necessary in this case, but because they were necessary in the metabolism experiments.

A solution containing the following was prepared from the stock solutions: 5 cc. of 2 per cent histidine dichloride, 4 cc. of 1 per cent histamine dichloride, 2 cc. of 1 per cent imidazole propionic acid, 2 cc. of 1 per cent imidazole acetic acid, and 2 cc. of 1 per cent methyl imidazole. This was mixed with 10 cc. of 2 per cent ammonium chloride, 4 cc. of glycerol, 100 cc. of Salt Medium 1[1], and 76 cc. of water in a 300 cc. Pyrex Florence flask.

Hydrogen Ion Concentration.—1 cc. of the finished solution was treated with 0.10 cc. of a 0.01 per cent solution of phenolsulfonephthalein and the color produced compared with a set of standard tubes.[9] In this way the original solution was found to have a pH of 6.8.

[9] Furnished by Hynson, Westcott, and Dunning, Baltimore, Md.

Filtration.—The remaining solution was forced through a Berkefeld filter. The flask and filter were washed free from imidazoles with 200 cc. of distilled water. The filtrate was treated with 1 cc. of 95 per cent sulfuric acid, transferred to a 200 cc. glass dish and evaporated on the water bath until salts began to crystallize. The residue was carefully transferred to a graduated precision cylinder with enough water to give a final volume of exactly 25 cc. This will be referred to as the test solution.

Total Color Value.—2.50 cc. of the test solution were diluted to 100 cc. in a volumetric flask. Of this solution 0.10 cc. had a color value equivalent to 17.7–17.9 mm. (CR-MO) the color being slightly too red for the standard indicator. 0.10 cc. of this solution should contain

	gm.		*mm.*	
Histidine dichloride	0.00001	Color value	5.0	(CR-MO)
Histamine "	0.000004	" "	3.0	"
Methyl imidazole	0.000002	" "	4.9	"
Imidazole propionic acid	0.000002	" "	2.7	"
" acetic "	0.000002	" "	2.3	"
Theory			17.9	"

The check between fact and theory is perfect which adds weight to the statement made in Section I. That so close a check should have been obtained is surprising when the different rates of color development for these five substances are considered. The results obtained here show that *imidazoles are not adsorbed by a Berkefeld filter.*

Methyl Imidazole (Method A).—The remaining 98 cc. of the above solution were transferred to a glass dish, treated with 5 cc. of a M Na_2CO_3 solution, and evaporated to a volume of about 5 cc. 50 cc. of water were then added and the solution reevaporated to a volume of 5 cc. Finally 5 cc. of 37 per cent HCl were added carefully and the liquid was evaporated until all the water and acid had volatilized. The residue was dissolved in water and diluted to exactly 98 cc.

Of this solution 0.10 cc. had a color value equivalent to 13.2 mm. (CR-MO). $17.9 - 13.2 = 4.7$ mm. (CR-MO) or 5.2 mm. (CR)[10] was the color value due to methyl imidazole. This, by

[10] Koessler, K. K., and Hanke, M. T., *J. Biol. Chem.*, 1919, xxxix, 497.

table, is equivalent to 0.0193 gm. of methyl imidazole for the entire test solution which is 97 per cent of that actually introduced.

Separation of the Histamine from the Histidine Fraction.—10 cc. of the test solution were measured into the extraction tube. 3 gm. of solid sodium hydroxide were added to the solution and the mixture cooled in tap water while the alkali was dissolving. The strongly alkaline aqueous liquid was extracted six times with prepared amyl alcohol in the manner described in Section III.

Histidine Fraction.—The alkaline liquid was carefully transferred to a graduated precision cylinder with 10 cc. of 20 per cent N HCl. The liquid, which was still alkaline to litmus paper, was rendered faintly acid by adding 95 per cent H_2SO_4 drop by drop. Water was then added to the cooled solution to bring its volume up to exactly 25 cc. About 10 cc. of chloroform were finally added[6] and the mixture was vigorously agitated.

Color Value.—6.25 cc. of the clear aqueous layer were diluted to 100 cc. in a precision cylinder and vigorously agitated with about 20 cc. of chloroform. Of the clear aqueous layer, 0.10 cc. had a color value equivalent to 11.0 mm. (CR). In this case the (CR) indicator gave a better match than the (CR-MO) indicator. 11.0 mm. (CR) are equivalent to 10.0 mm. (CR-MO).[10] The color value of this solution should have been

	gm.		*mm.*	
Histidine dichloride	0.00001	Color value	5.0	(CR-MO)
Imidazole propionic acid	0.000002	" "	2.7	"
" acetic "	0.000002	" "	2.3	"
Theory			10.0	"

The agreement was perfect. This proves that histidine, imidazole propionic acid, and imidazole acetic acid are quantitatively retained by the alkali and appear together in the histidine fraction. Of these three substances only one, histidine, can be determined directly. We have been unable to devise a method for estimating either imidazole acetic or propionic acid separately, so that if both should appear in an unknown it would be impossible to differentiate between them. Their combined quantity can be determined by subtracting from the total that part of the color value that is due to histidine, as calculated from the Van Slyke determination, and calculating the excess color value to either imidazole propionic or acetic acid.

Amino Nitrogen Determination (Histidine).—The amino nitrogen in 5 cc. of the histidine fraction was determined by the Van Slyke method using the micro-apparatus. There was thus obtained 0.88 cc. of N_2 at 16° and 749 mm., which is equivalent to 0.1024 gm. of histidine dichloride for the entire test solution. This is 102.4 per cent of the amount originally introduced which is within the limit of experimental error for so small a quantity of substance. This proves that the ammonia present in the original test solution has passed into the amyl alcohol extract so that no special precautions need be taken to free the histidine fraction from ammonia. This statement has been amply verified by experiments described in the following paper. It need hardly be said that this method will be an accurate index of the quantity of histidine in the test solution only when other amino-acids are absent.

The Histamine Fraction.—The combined amyl alcohol extracts were extracted with 1.0 N H_2SO_4 as has already been described under Section III. The solution finally obtained was too concentrated to make an accurate determination possible, so 12.5 cc. of it were diluted to 50 cc. in a volumetric flask.

Of this diluted solution 0.10 cc. had a color value equivalent to 8.7 mm. (CR) or 7.9 mm. (CR-MO) and 0.20 cc. had a color value equivalent to 17.4 mm. (CR) or 15.8 mm. (CR-MO).

If the methyl imidazole and histamine had been entirely extracted by the amyl alcohol, 0.10 cc. of this solution should have had a color value equivalent to 7.9 (CR-MO). The check is perfect.

Removal of Ammonia and Methyl Imidazole by Evaporation with Alkali.—The entire histamine fraction was recombined and evaporated in a glass dish with 5 cc. of a M Na_2CO_3 solution until a dry residue was obtained. This was then dissolved in water, treated with an excess of 37 per cent HCl, and the resulting solution again evaporated to remove the water and acid entirely. The solid so obtained was transferred to a precision cylinder with enough water to give a final volume of 20 cc.

Amino Nitrogen Determination (Histamine).—Of the above solution, 5 cc. gave 0.56 cc. of N_2 at 25° and 747 mm., which is equivalent to 0.04015 gm. of histamine dichloride for the entire test solution and is 100.4 per cent of that actually introduced. Although this method works ideally when pure substances are

STUDIES ON PROTEINOGENOUS AMINES.

IV. THE PRODUCTION OF HISTAMINE FROM HISTIDINE BY BACILLUS COLI COMMUNIS.

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INTRODUCTION.

β-imidazolylethylamine (4-β-aminoethylglyoxaline), or histamine as it is appropriately called, was first obtained by Windaus and Vogt[1] in 1907 through synthesis from imidazole propionic acid. As a product of putrefaction of histidine it was first isolated by Ackermann[2] in 1910, who inoculated a synthetic medium containing histidine hydrochloride, peptone, glucose, and the necessary mineral salts with a piece of beef pancreas which had been allowed to putrefy for 24 hours. Aside from histamine, Ackermann was also able to separate a small quantity of imidazole propionic acid from the putrefaction mixture. In the same year the remarkale physiological properties of histamine were discovered independently by Barger and Dale,[3] and by Kutscher,[4] who found that it was one of the active principles of ergot. The physiological action of the base was then studied in great detail by many investigators, especially by Dale[5] and his coworkers. In 1911 Barger and Dale[6] obtained histamine from the mucosa of the small intestine of the ox and ascribed the depressor action of secretin, at least in part, to this base. The question whether histamine is a normal product of the intestinal mucosa or whether it is found there as a product of the bacterial decomposition of proteins containing histidine was decided in favor of the latter assumption by Mellanby and Twort[7] who isolated, from

[1] Windaus, A., and Vogt, W., *Ber. chem. Ges.*, 1907, xl, 3691.

[2] Ackermann, D., *Z. physiol. Chem.*, 1910, lxv, 504.

[3] Barger, G., and Dale, H. H., *Proc. Physiol. Soc.*, 1910, xxvi, 128; *Zentr. Physiol.*, 1910–11, xxiv, 885; *J. Chem. Soc.*, 1910–11, xcvii, 2592.

[4] Kutscher, F., *Centr. Physiol.*, 1910–11, xxiv, 163.

[5] Dale, H. H., and Laidlaw, P. P., *J. Physiol.*, 1910–11, xli, 318; 1911–12, xliii, 182.

[6] Barger, G., and Dale, H. H., *J. Physiol.*, 1910–11, xli, 499.

[7] Mellanby, E., and Twort, F. W., *J. Physiol.*, 1912–13, xlv, 53.

the intestinal contents of the guinea pig, a bacillus of the colon-typhoid group capable of converting histidine into histamine. In the same year Berthelot and Bertrand[8] isolated a bacillus from the human intestine that resembled Friedländer's pneumobacillus in all its morphological and chemical characteristics, but which had the additional faculty of decarboxylating histidine to histamine. They named this organism *Bacillus aminophilus intestinalis.*

This apparently simple chemical process, the decarboxylation of amino-acids to exceedingly potent substances of amine structure, is of great theoretical and practical interest. The relation of this problem to the general nutrition of bacteria, to the metabolism of amino-acids in the mammalian organism, to the pathology and pharmacology of the smooth muscle fiber system, and to the chemical constitution of the products of the glands of internal secretion, mark it as a fundamental inquiry of biology. The systematic investigation of these problems has been under way in this laboratory for the past 4 years.

Theoretical considerations and preliminary experiments had convinced us that the ability to decarboxylate amino-acids to amines could not be restricted to one or two species of bacteria but might well be a fairly common property of microorganisms. It soon became apparent that to attack the problem successfully it would be necessary to devise quantitative chemical methods for the determination and separation of the various proteinogenous amines as well as the other possible products resulting from the catabolism of the amino-acids. The preparation of the amino-acids, amines, intermediates, and by-products was an obvious preliminary to the attack on the bacterial metabolism experiments. We have already reported in detail on the preparation of β-imidazolylethylamine.[9] The preceding two articles in this *Journal* describe the quantitative methods used for the separation and estimation of imidazoles. It is the object of this communication to show what the optimum conditions are for the conversion of histidine to histamine by the colon bacillus. It is our intention to report a series of experiments in the near future that will show which of the common pathogenic and non-patho-

[8] Berthelot, A., and Bertrand, D.-M., *Compt. rend. Acad.*, 1912, cliv, 1643, 1826.

[9] Koessler, K. K., and Hanke, M. T., *J. Am. Chem. Soc.*, 1918, xl, 1716.

genic bacteria possesses the faculty of decarboxylating histidine. Work is also under way to apply similar methods to a study of the decarboxylation of tyrosine and tryptophane.

EXPERIMENTAL.

Foreword.

We have divided the experimental material presented in this article into three parts.

Part I contains a description of seventeen experiments that show the effect of *the composition of the medium* upon the metabolism of histidine by the colon bacillus.

The experiments described in Part II were carried out to ascertain *how the amount of histamine produced varied with the duration of the experiment.*

Part III contains a description of four experiments that seem to throw some light on *the causal relation between the formation of histamine and the hydrogen ion concentration of the medium.*

Part I. Effect of the Composition of the Medium upon the Metabolism of Histidine by the Colon Bacillus.

Preparation of Stock Solutions.

Stock Histidine Solution.—Vacuum-dried, 100 per cent histidine dichloride—4.0000 gm.—was dissolved in water and diluted to exactly 200 cc. in a volumetric flask.

Stock Ammonium Chloride Solution.—J. T. Baker's 97.4 per cent ammonium chloride—2.0000 gm.—was dissolved in water and diluted to exactly 100 cc.

Stock Potassium Nitrate Solution.—Kahlbaum's 100 per cent potassium nitrate—2.0000 gm.—was dissolved in water and diluted to exactly 100 cc.

Salt Medium 1.—0.30 gm. of potassium chloride.
8.00 " of potassium dihydrogen phosphate.
24.00 " of sodium chloride.
0.40 " " sulfate (anhydrous).
8.00 " " bicarbonate.
0.20 " calcium chloride (anhydrous).

These amounts were dissolved in water and diluted to exactly 2,000 cc. When properly prepared the salt medium should contain no precipitate.

Mode of Preparing Completed Media.

The most complex media were Nos. 15 and 16. They were prepared as follows:

10 cc. each of the stock histidine and ammonium chloride solutions and 5 cc. of stock potassium nitrate solution were measured, by means of normal pipettes, into a 300 cc. Pyrex Florence flask. 4 cc. of glycerol, measured by graduate, were washed into the Pyrex flask with 71 cc. of water. Exactly 100 cc. of salt medium were then added.

Each of the seventeen completed media had a final volume of 200 cc. before sterilization, and contained exactly 0.2000 gm. of histidine dichloride.

Nos. 1 and 2 contained	10 cc.	of stock 2 per cent histidine dichloride,
	100 "	" salt medium,
	90 "	" water.
Nos. 3 and 4 contained	10 cc.	of stock 2 per cent histidine dichloride,
	10 "	" " 2 " " ammonium choride,
	100 "	" salt medium,
	80 "	" water.
Nos. 5 and 6 contained	10 cc.	of stock 2 per cent histidine dichloride,
	4 "	" glycerol,
	100 "	" salt medium,
	86 "	" water.
Nos. 7 and 8 contained	10 cc.	of stock 2 per cent histidine dichloride,
	10 "	" " 2 " " ammonium chloride,
	4 "	" glycerol,
	100 "	" salt medium,
	76 "	" water.
Nos. 9 and 10 contained	10 "	" stock 2 per cent histidine dichloride,
	5 "	" " 2 " " potassium nitrate,
	100 "	" salt medium,
	85 "	" water.
Nos. 11 and 12 contained	10 cc.	of stock 2 per cent histidine dichloride,
	5 "	" " 2 " " potassium nitrate,
	10 "	" " 2 " " ammonium chloride,
	100 "	" salt medium,
	75 "	" water.
Nos. 13 and 14 contained	10 cc.	of stock 2 per cent histidine dichloride,
	5 "	" " 2 " " potassium nitrate,
	4 "	" glycerol,
	100 "	" salt medium,
	81 "	" water.

Nos. 15 and 16 contained 10 cc. of stock 2 per cent histidine dichloride,
5 " " " 2 " " potassium nitrate
10 " " " 2 " " ammonium chloride,
4 " " glycerol,
100 " " salt medium,
71 " " water.

No. 17 contained 10 cc. of stock 2 per cent histidine dichloride,
5 " " " 2 " " potassium nitrate,
10 " " " 2 " " ammonium chloride,
5 gm. of crystalline glucose,
100 cc. of Salt Medium 1,
70 " " distilled water.

Sterilization.

The seventeen flasks were stoppered with cotton and sterilized by autoclaving for 1 hour at 10 pounds pressure. Calcium phosphate precipitated as carbon dioxide escaped.

Hydrogen Ion Concentration before Inoculation.

This was determined by the method described on page 534. Each of the sixteen completed media had a pH of 7.35 before inoculation.

Inoculation with Bacillus coli communis.

The culture used was a laboratory stock culture, originally isolated from human feces.

The 24 hours growth on sixteen agar slants was transferred with sterile salt solution to a sterile 25 cc. graduated precision cylinder. The emulsion was then diluted to 25 cc. with sterile salt solution.

Method for Counting Bacteria.

Of the well mixed emulsion, 1 cc. was diluted to 250 cc. Of this dilute suspension, 0.01 cc. was spread out smoothly over an area of 10 sq. cm. and the bacteria stained with 5 per cent aqueous fuchsin. They were then counted by the method of Breed and Brew[10] which showed that about 9,000,000,000 bacteria were

[10] Breed, R. S., and Brew, J. D., *New York Agric. Exp. Sta., Tech. Bull. 49*, 1916.

present in every cc. of the original bacterial emulsion. Exactly 1 cc. of the original emulsion was transferred to each of the sixteen flasks.

Incubation.

The nine odd numbered flasks were placed in an incubator. This is the aerobic series. The eight even numbered flasks were placed in a large vacuum desiccator with a dish containing pyrogallic acid and sodium hydroxide. The desiccator was completely exhausted, placed in the incubator, and reexhausted once a day to insure against leakage which was very slight. This is the anaerobic series.

The mixtures were incubated for 14 days.

Growth of Bacteria.

Judging from the degree of turbidity of the solutions, the bacteria grew best in Flasks 7, 8, 13, 14, 15, and 16. A very good growth was also obtained in Flasks 5, 6, 11, and 17. Flasks 12, 9, 10, 3, 4, 1, and 2 had the least growth in descending order.

The aerobic solutions were pale yellow to green, the anaerobic were colorless to very pale yellow.

Filtration.

The contents of each flask were forced through a clean 2.5 inch Berkefeld filter. The flask and filter were then washed free from imidazoles with about 200 cc. of distilled water. The filtrate obtained was always perfectly clear.

Hydrogen Ion Concentration after Incubation and Filtration.

1 cc. of the undiluted filtrate was used for the pH determination. The following results were obtained.

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
pH	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*
8.3	7.6	8.2	7.4	7.15	7.45	>6.6	>6.6	8.4	8.0	8.3	7.9	>6.6	>6.6	>6.6	>6.6	>6.6

Test Solution.

The filtrate and washings were treated with 1 cc. of 95 per cent H_2SO_4 and evaporated in a 200 cc. glass dish until salts began to crystallize. This residue was then transferred to a precision cylinder with enough water to give a final volume of 25 cc. This liquid will be referred to as the test solution.

Total Color Value.

2.50 cc. of the test solution were diluted to exactly 100 cc. Portions of this diluted solution were then used for the colorimetric determinations. The results obtained are given in Table I.

TABLE I.
Total Color Value.

Flask No.	Diluted solution.	Color value of (CR-MO) indicator.		Relation of total color value to the original color value.
	cc.	*mm.*		*per cent*
1	1.0	2.0	Brown, match poor.	2.0
2	0.10	6.6		66.0
	0.20	13.2		
3	1.0	3.5	Brown, match poor.	3.5
4	0.10	10.1		98.0
	0.20	20.3		
5	0.10	9.0		90.0
	0.20	18.0		
6	0.10	10.4		104.0
	0.20	20.8		
7	0.10	9.4		94.0
	0.20	18.8		
8	0.10	10.0		100.0
	0.20	20.0		
9	1.0	3.7	Brown.	3.7

TABLE I.—*Concluded.*

Flask No.	Diluted solution.	Color value of (CR-MO) indicator.		Relation of total color value to the original color value.
	cc.	*mm.*		*per cent*
10	0.10	9.1		91.0
	0.20	18.2		
11	1.0	2.7	Brown.	2.7
12	0.10	10.0		100.0
	0.20	20.0		
13	0.10	10.0		100.0
	0.20	20.0		
14	0.10	9.7		97.0
	0.20	19.4		
15	0.10	10.0		100.0
	0.20	20.0		
16	0.10	10.0		100.0
	0.20	20.0		
17	0.10	9.3		93.0
	0.20	16.0		

Separation of the Histamine from the Histidine Fraction.

This separation was conducted on 10 cc. of the test solution exactly as has already been described on page 534. No separation was conducted on Flasks 1, 3, 9, and 11, because the total color value determination showed that the imidazole ring had been almost completely destroyed in these cases.

Histidine Fraction.—Of the histidine fraction, whose final volume was 25 cc., 6.25 cc. were diluted to 100 cc. for the color value determination, and 5 cc. portions were analyzed for amino nitrogen, all the steps being duplicates of the process already described. The results are given in Table II.

TABLE II.

Histidine Fraction.

Flask No.	Solution used.	Color value of (CR-MO) indicator.	Color value of histidine fraction as histidine.	Introduced histidine recovered unchanged (Van Slyke method) from 5 cc. of test solution.	Histidine converted into imidazole propionic acid (by difference).
	cc.	*mm.*	*per cent*		*per cent*
2	0.10 0.20	6.4 12.7	64	1.13 cc. N_2 at 20° and 748 mm. 0.1290 gm. histidine Cl_2. **64.5** %	0
4	0.10 0.20	10.1 19.8	98	1.80 cc. N_2 at 22.5° and 741 mm. 0.2008 gm. histidine Cl_2. **100.4** %	0
5	0.10 0.20	7.9 15.8	79	1.42 cc. N_2 at 20° and 752 mm. 0.1629 gm. histidine Cl_2. **81.5** %	0
6	0.10 0.20	7.4 14.8	74	1.05 cc. N_2 at 25° and 745 mm. 0.1162 gm. histidine Cl_2. **58.1** %	8.15
7	0.10 0.20	4.6 9.2	46	0.80 cc. N_2 at 20° and 754 mm. 0.092 gm. histidine Cl_2. **46.0** %	0
8	0.10 0.20	6.4 12.7	64	1.18 cc. N_2 at 24° and 742 mm. 0.131 gm. histidine Cl_2. **65.5** %	0
10	0.10 0.20	8.9 17.8	89	1.60 cc. N_2 at 25° and 753 mm. 0.1792 gm. histidine Cl_2. **89.6** %	0
12	0.10 0.20	9.8 19.7	98	1.75 cc. N_2 at 20° and 750 mm. 0.20 gm. histidine Cl_2. **100.0** %	0
13	0.10 0.20	4.0 8.0	40	0.70 cc. N_2 at 23° and 753 mm. 0.0791 gm. histidine Cl_2. **39.5** %	0

TABLE II—*Concluded.*

Flask No.	Solution used.	Color value (CR–MO) indicator.	Color value of histidine fraction as histidine.	Introduced histidine recovered unchanged (Van Slyke method) from 5 cc. of test solution.	Histidine converted into imidazole propionic acid (by difference).
	cc.	*mm.*	*per cent*		*per cent*
14	0.10	9.0	90	1.60 cc. N_2 at 23° and 753 mm.	0
	0.20	18.1		0.1807 gm. histidine Cl_2.	
				90.4 %	
15	0.10	4.5	45	0.85 cc. N_2 at 23° and 754 mm.	0
	0.20	9.0		0.0961 gm. histidine Cl_2.	
				48.0 %	
16	0.10	7.5	75	1.35 cc. N_2 at 20° and 753 mm.	0
	0.20	15.0		0.1549 gm. histidine Cl_2.	
				77.4 %	
17	0.10	5.3	53	1.06 cc. N_2 at 24° and 737 mm.	0
	0.20	8.2		0.1168 gm. histidine Cl_2.	
				58.4 %	

Discussion.—The colors produced by the histidine fraction were indistinguishable from those produced by pure histidine excepting No. 6. In this case the color produced was slightly too pink for the indicator which would suggest the presence of imidazole propionic, acetic, or lactic acid. The color value of No. 6 was equivalent to 74 per cent of the original color value. The Van Slyke determination showed that only 58 per cent of the histidine originally introduced was still present. It was quite clear, therefore, that in this one case a part of the histidine had been deaminized. We did not determine the character of the product but assumed it to be imidazole propionic acid because this substance has actually been isolated by Ackermann[2] as a product of the bacterial metabolism of histidine. If 58 per cent of the original color value is assumed to be due to histidine, this would be equivalent to 5.8 mm. (CR-MO) for 0.10 cc. of the solution. The difference between this and the observed color value—1.6 mm. (CR-MO)—would be due to imidazole propionic acid. This is equivalent to 0.01 gm. of imidazole propionic acid for the entire test solution

which shows that 8.15 per cent of the histidine was converted into this substance.

We wish to call attention here, to the close check between the histidine values obtained by the Van Slyke and the colorimetric methods. The entire significance of this agreement will be discussed in detail at the end of the article. This agreement was not so close in No. 17 where glucose was used in place of glycerol. The products formed by the metabolism of glucose interfere with the color production. This is proved quite clearly by the discrepancy between the color values obtained from 0.20 and 0.10 cc. of the solution. Since 0.10 cc. of the solution had a color value equivalent to 5.3 mm. (CR-MO), 0.20 cc. should have had a color value equivalent to 10.6 mm. The value actually obtained was 8.2 mm. which shows that the color production had been seriously interfered with. A smaller interference would be expected from 0.10 cc. because less of the objectionable substances were introduced. For that reason the calculations were based upon the color value for 0.10 cc.

Histamine Fraction.—This was obtained by extracting the combined amyl alcohol extracts with 1.0 N H_2SO_4 as has already been described under Sections III and VII of the previous paper. The final volume of the neutral solution was always 100 cc. The colorimetric determinations were made on this solution without further dilution. That portion of the histamine fraction which was not used for the colorimetric determinations—99 cc. or more—was treated with 5 cc. of M Na_2CO_3 solution and evaporated to dryness on the water bath. An excess of 37 per cent HCl was then added and the liquid reevaporated to remove the water and acid entirely. The residue was transferred to a graduated precision cylinder with enough water to give a final volume of 25 cc. This concentrated solution, which was now free from ammonia and volatile amines, was used for the following determinations.

1. An Amino Nitrogen Determination.—This was carried out in only a few cases because the presence of about 10 per cent of histamine was regularly indicated by the Van Slyke method when the colorimetric determination showed this substance to be entirely absent. We have found more recently (see Sections II and III of Paper III) that the Van Slyke method can be used for the estimation of histamine when that substance is produced in large

amounts. The entire significance of these facts will be discussed in detail at the end of this section.

2. A Color Value Determination.—In case this determination agreed with the previous determination it served as a check, or if it had been lower than the previous determination it would have *indicated* the presence of methyl imidazole. The redetermined color value always agreed perfectly with that previously determined, which proved that methyl imidazole was regularly absent.

3. A biological verification of the presence of histamine.

4. A chemical verification of the presence of histamine.

The results are given in Table III.

TABLE III.

Histamine Fraction.

Flask No.	Amount of solution used.	Color value of (CR-MO) indicator.	Histamine.		Histidine converted into methyl imidazole by Methods B and C.
			In test solution.	Histidine converted.	
	cc.	*mm.*	*gm.*	*per cent.*	*per cent*
2	1.0	3.0 Brown.	0.0009	0.45	None.
4	1.0	4.0 Too yellow.	0.00125	0.6	"
5	1.0	4.0 " "	0.00125	0.6	"
6	1.0	3.8 " "	0.0012	0.6	"
7	0.05	11.2	0.075	46.4	"
	0.10	22.2			
8	0.10	6.6	0.0221	13.7	"
	0.20	13.1			
10	1.0	3.8 Too yellow.	0.0012	0.6	"
12	1.0	4.0 " "	0.00125	0.6	"
13	0.05	12.4	0.0825	51.0	"
	0.10	24.7			
14	0.20	6.4	0.01075	6.7	"
	0.40	12.8			
15	0.05	12.1	0.0807	50.0	"
	0.10	24.2			
16	0.10	7.5	0.025	15.5	"
	0.20	15.0			
17	0.10	12.6	0.0425	26.3	"
	0.20	25.3			

Discussion.—The color value of the histamine fraction never was nil. Values lower than 4.0 mm. (CR-MO) for 1.0 cc. of the solution were due to *histidine* not *histamine* (see p. 525). The constancy of this value in those cases where histamine was absent is striking. The color development for histamine is characteristic of that substance and is hard to confuse with that of either histidine or methyl imidazole. In those seven cases where histamine is recorded as present, the color was identical with that obtained from pure histamine. Further proof of the correctness of this method as a means of determining histamine is given below.

Biological Verification of the Presence of Histamine.

A portion of the solution, which had been shown to contain 1 mg. of histamine dichloride by the colorimetric method, was injected into the femoral vein of a dog that had been previously anesthetized and prepared so that a blood pressure and respiratory tracing could be obtained at the same time. Figs. 1 and 2 are representative tracings. Similar tracings were obtained from Samples 7, 8, 13, 14, and 17. The entire Solution 5, which can be taken as a representative of those solutions in which a color value of 4.0 mm. (CR-MO) per 1 cc. was obtained, was injected. There was no apparent change either in blood pressure or respiration. The curve for No. 15 shows an effect that is usually not obtained. The injection was started and interrupted after a fraction of a drop of the solution had entered the circulation. Although this small quantity of substance produced a marked bronchial spasm, it raised the blood pressure very slightly. The subsequent injection of the remainder of the mg. of substance produced the customary drop in blood pressure, together with an intense bronchial spasm.

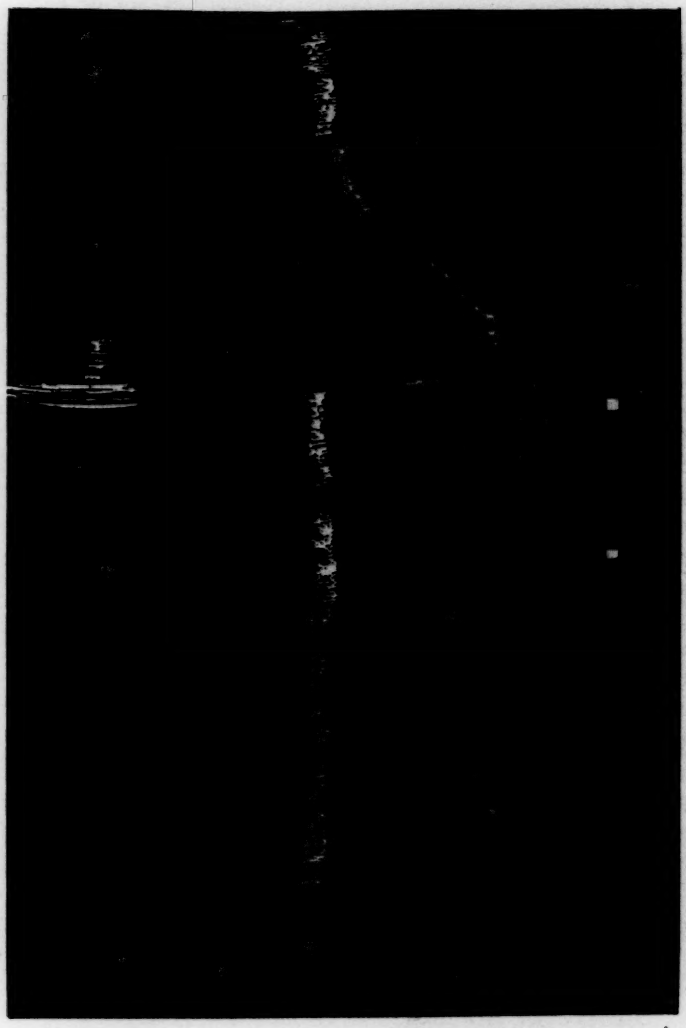


FIG. 1. Respiratory and blood pressure tracings obtained by injecting 0.60 cc. of Histamine fraction No. 15—1.0 mg. of histamine dichloride by colorimetric determination—into the femoral vein of a dog.

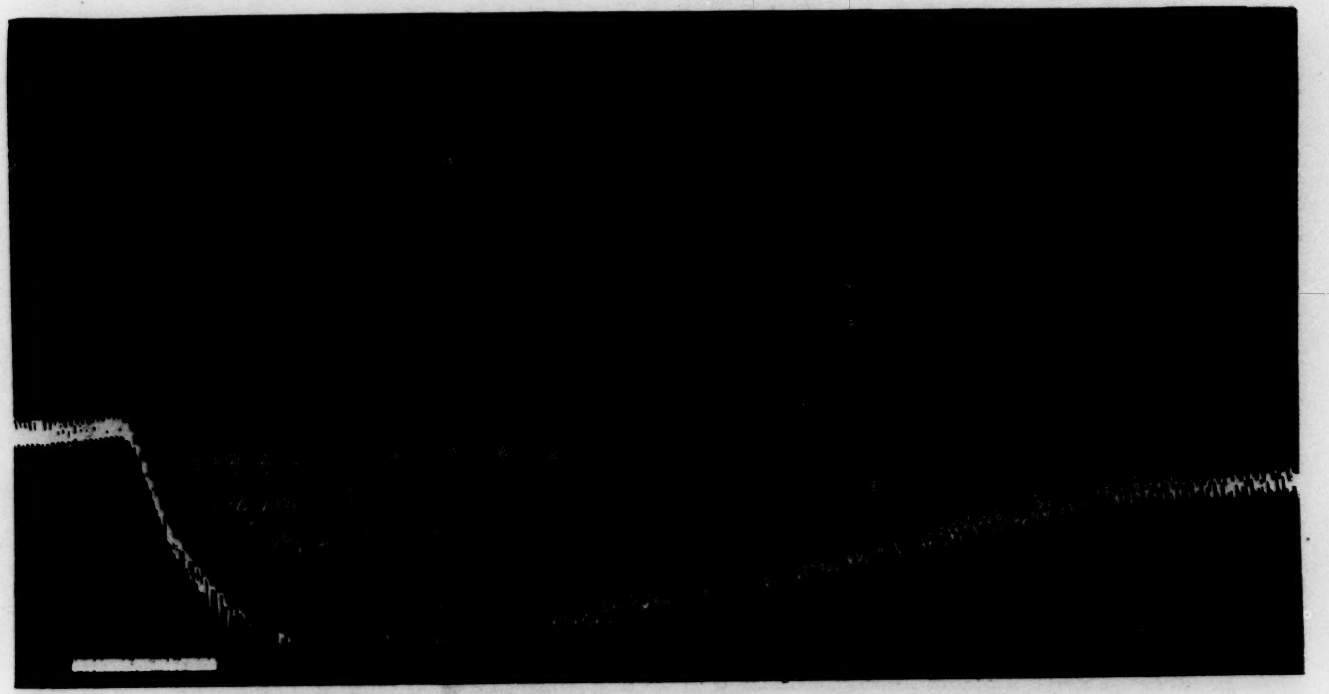


FIG. 2. Respiratory and blood pressure tracings obtained by injecting 2 cc. of Histamine fraction No. 16—1.0 mg. of histamine dichloride by colorimetric determination—into the femoral vein of a dog.

Chemical Verification of the Presence of Histamine.

The remainder of Solutions 7, 8, 13, 14, 15, and 16, whose combined histamine content was 0.10 gm. by colorimetric determination, was united, made strongly alkaline with sodium hydroxide, and extracted with amyl alcohol in the customary manner. The amyl alcohol was then extracted exhaustively with 6 N HCl. The acid extracts were combined and evaporated on the water bath. An entirely solid, pale brown residue was left which was largely sodium chloride. A melting point determination was carried out on a portion of it. A slight softening occurred at 195°. No further change was noticeable until the temperature had risen to 242° (corrected) when an abrupt softening occurred. The solid did not, of course, melt completely because of the presence of sodium chloride. The recorded melting point of histamine dichloride is 244–246° (corrected.)

The solid gave 0.230 gm. of dipicrate in the form of rhombic leaflets, M.P. 238–240°, from 100 cc. of water. This is the recorded melting point of histamine dipicrate. Some of this solid was mixed with an equal portion of pure synthetic histamine dipicrate. The melting point of the mixture was 238–240°. This proves conclusively that the solid was histamine dipicrate.

A further proof of the quantitative exactness of the colorimetric method can be cited. 0.10 gm. of histamine dichloride should give 0.31 gm. of dipicrate. We have found that the solubility of histamine dipicrate in 100 cc. of water is 0.0716 gm. at 8°, 0.077 at 20°, and 1.4 at 95°. Since this picrate was filtered from a solution whose temperature was about 10°, 0.07 gm. of dipicrate was left in the 100 cc. of solvent. Since 0.23 gm. of dipicrate was actually isolated, 0.30 gm. must have been formed in all which agrees very well with the 0.31 gm. demanded by the theory.

Determination of Ammonia and Total Amino Nitrogen in the Test Solution.

Ammonia was determined by the method given in Section VII of Paper III.

The amino nitrogen determinations referred to here were carried out only on those solutions whose total color value was almost nil. In these cases 10 cc. of the test solution were evapo-

rated on the water bath with 5 cc. of M Na_2CO_3 solution until the liquid was free from ammonia and volatile amines. (These solutions always had a very disagreeable amine-like odor when alkaline which disappeared after the liquid had been heated for some time.) An excess of hydrochloric acid was then added and the liquid reevaporated until all the water and acid had disappeared. The solid was transferred to a precision cylinder with enough water to give a final volume of 10 cc. 5 cc. of this solution were then used for the amino nitrogen determinations. The results are given in Tables IV and V.

TABLE IV.

Ammonia and Total Amino Nitrogen.

Flask No.	0.1 N HCl neutralized by NH_3 from 5 cc. of test solution.	Total nitrogen as NH_3.*	N_2 from 5 cc. of test solution (Van Slyke method).			Total N as amino N.
			Amount.	Temperature.	Pressure.	
	cc.	*per cent*	*cc.*	°C.	*mm.*	*per cent*
1	3.51	66.8	1.75	23	753	13.2
2	1.52	28.9				
3	10.38	82.5	1.97	19	744	6.28 (15.0 per cent of histidine N).
4	6.75	53.8				
5	0.10	1.9				
6	0.10	1.9				
7	3.50	27.9				
8	4.46	35.5				
9	3.95	75.0	1.25	20	748	9.5 (also histidine N).
10	0.50	9.5				
11	8.30	66.2	1.66	20	756	5.35 (12.8 per cent of histidine N).
12	5.80	46.2				
13	0.10	1.9				
14	0.90	17.1				
15	5.68	45.3				
16	6.48	51.7				
17	8.15	65.0				

* N_2 in KNO_3 was ignored.

TABLE

Summary of

Flask No.	Composition of completed medium; incubated 14 days at 37°.	Total color value of test solution as histidine Cl_2.* (0.20 gm. = 100 %.)	Color value of histidine fraction as histidine Cl_2.* (0.20 gm. = 100 %.)	Unchanged histidine (Van Slyke method) with 5 cc. of test solution.	Histidine converted into imidazole propionic acid (by difference).
					per cent
1. Aerobic.	10 cc. 2 % histidine Cl_2. 100 cc. Salt Medium 1. 90 cc. distilled water.	1.0 cc. = 0.02 mm. Color brown; match poor. **2%**			0
2. Anaerobic.	Same as No. 1.	0.1 cc. = 6.6 mm. 0.2 " = 13.2 " Match perfect. **66.0 %**	0.1 cc. = 6.4 mm. 0.2 " = 12.7 " Match perfect. **64.0 %**	1.13 cc. N_2 at 20° and 748 mm. 0.1290 gm. histidine Cl_2. **64.5 %**	0
3. Aerobic.	10 cc. 2 % histidine Cl_2. 100 cc. Salt Medium 1. 10 cc. 2 % NH_4Cl. 80 cc. distilled water.	1.0 cc. = 3.5 mm. Color brown; match poor. **3.5 %**			0
4. Anaerobic.	Same as No. 3.	0.1 cc. = 10.1 mm. 0.2 " = 20.3 " Match perfect. **98.0 %**	0.10 cc. = 10.1 mm. 0.20 " = 19.8 " Match perfect. **98.0 %**	1.8 cc. N_2 at 22° and 741 mm. 0.2008 gm. histidine Cl_2. **100.4 %**	0
5. Aerobic.	10 cc. 2 % histidine Cl_2. 4 cc. glycerol. 100 cc. Salt Medium 1. 86 cc. distilled water.	0.1 cc. = 9.0 mm. 0.2 " = 18.0 " Match perfect. **90.0 %**	0.10 cc. = 7.9 mm. 0.20 " = 15.8 " Match perfect. **79.0 %**	1.42 cc. N_2 at 20° and 752 mm. 0.1629 gm. histidine Cl_2. **81.5 %**	0

Results. Part I.

Color value of histamine fraction.*	Histidine converted into histamine (colorimetric determination).	Histidine converted into methyl imidazole (Methods B and C).	Amount of 0.1 N HCl neutralized by ammonia from entire test solution.	Total N as NH_3 (N_2 in KNO_2 ignored).	Total N as amino nitrogen with 5 cc. of test solution.	Reaction.	
						Before incubation.	After incubation.
		per cent	*cc.*	*per cent*		*pH*	*pH*
	Histamine absent.	0	17.55	66.8	1.75 cc. N_2 at 23° and 753 mm. 13.2 %.	7.35	8.3
1.0 cc. = 3.0 mm. Color too yellow; match poor.	≏ 0.0009 gm. histamine in test solution. 0.45 % Histamine absent.	0	7.6	28.9		7.35	7.6
	Histamine absent.	0	51.9	82.5	1.97 cc. N_2 at 19° and 744 mm. 6.28 % total N (15.0 % histidine N).	7.35	8.2
1.0 cc. = 4.0 mm. Color too yellow; match poor.	≏ 0.00125 gm. histamine in test solution. 0.60 % Histamine absent.	0	33.75	53.8		7.35	7.5
1.0 cc. = 4.0 mm. Color too yellow; match poor.	≏ 0.00125 gm. histamine in test solution. 0.60 % Histamine absent.	0	0.50	1.9		7.35	7.15

Flask No.	Composition of completed medium; incubated 14 days at 37°.	Total color value of test solution as histidine Cl_2.* (0.20 gm. = 100 %.)	Color value of histidine fraction as histidine Cl_2.* (0.20 gm. = 100 %.)	Unchanged histidine (Van Slyke method) with 5 cc. of test solution.	Histidine converted into imidazole propionic acid (by difference)
					per cent
6. Anaerobic.	Same as No. 5.	0.10 cc. = 10.4 mm. 0.20 " = 20.8 " Match perfect. **104.0 %**	0.10 cc. = 7.4 mm. 0.20 " = 14.8 " Too pink. **74.0 %**	1.05 cc. N_2 at 25° and 745 mm. 0.1162 gm. histidine Cl_2. **58.1 %**	8.1
7. Aerobic.	10 cc. 2 % histidine Cl_2. 10 cc. 2 % NH_4Cl. 4 cc. glycerol. 100 cc. Salt Medium 1. 76 cc. distilled water.	0.10 cc. = 9.4 mm. 0.20 " = 18.8 " Match perfect. **94.0 %**	0.10 cc. = 4.6 mm. 0.20 " = 9.2 " Match perfect. **46.0 %**	0.80 cc. N_2 at 20° and 754 mm. 0.092 gm. histidine Cl_2. **46.0 %**	0
8. Anaerobic.	Same as No. 7.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. **100.0 %**	0.10 cc. = 6.4 mm. 0.20 " = 12.7 " Match perfect. **64.0 %**	1.18 cc. N_2 at 24° and 742 mm. 0.131 gm. histidine Cl_2. **65.5 %**	0
9. Aerobic.	10 cc. 2 % histidine Cl_2. 5 cc. 2 % KNO_3. 100 cc. Salt Medium 1. 85 cc. distilled water.	1.0 cc. = 3.7 mm. Color brown; match poor. **3.7 %**			0
10. Anaerobic.	Same as No. 9.	0.10 cc. = 9.1 mm. 0.20 " = 18.2 " Match perfect. **91.0 %**	0.10 cc. = 8.9 mm. 0.20 " = 17.8 " Match perfect. **89.0 %**	1.60 cc. N_2 at 25° and 753 mm. 0.1792 gm. histidine Cl_2. **89.6 %**	0

Continued.

Results. Part I.

Color value of histamine fraction.*	Histidine converted into histamine (colorimetric determination).	Histidine converted into methyl imidazole (Methods B and C).	0.1 N HCl neutralized by ammonia from entire test solution.	Amount of total N as NH_3 (N_2 in KNO_3 ignored).	Total N as amino nitrogen with 5 cc. of test solution.	Reaction.	
						Before incubation.	After incubation.
		per cent	*cc.*	*per cent*		*pH*	*pH*
1.0 cc. = 3.8 mm. Color too yellow; match poor.	≏ 0.0012 gm. histamine in test solution. 0.6 % Histamine absent.	0	0.50	1.9		7.35	7.45
0.05 cc. = 11.2 mm. 0.10 " = 22.2 " Color identical with that obtained with histamine.	≏ 0.075 gm. histamine in test solution. **46.4** % of histamine present.	0	17.5	27.9		7.35	>6.6
0.10 cc. = 6.6 mm. 0.20 " = 13.1 " Color identical with that obtained with histamine.	≏ 0.0221 gm. histamine in test solution. **13.7** % of histamine present.	0	22.3	35.5		7.35	>6.6
	Histamine absent.	0	19.75	75.0	1.25 cc. N_2 at 20° and 748 mm. 9.5 % (ignoring KNO_3).	7.35	8.4
1.0 cc. = 3.8 mm. Color too yellow; match poor.	≏ 0.0012 gm. histamine in test solution. 0.6 % Histamine absent.	0	2.5	9.5		7.35	8.0

TABLE V

Summary of

Flask No.	Composition of completed medium; incubated 14 days at 37°.	Total color value of test solution as histidine Cl_2.* (0.20 gm. = 100 %.)	Color value of histidine fraction as histidine Cl_2.* (0.20 gm. = 100 %.)	Unchanged histidine (Van Slyke method) with 5 cc. of test solution.	Histidine converted into imidazole propionic acid (by difference).
					per cent
11. Aerobic.	10 cc. 2 % histidine Cl_2. 5 cc. 2 % KNO_3. 10 cc. 2 % NH_4Cl. 100 cc. Salt Medium 1. 75 cc. distilled water.	1.0 cc. = 2.7 mm. Color brown; match poor. **2.7 %**			0
12. Anaerobic.	Same as No. 11.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. **100.0 %**	0.10 cc. = 9.8 mm. 0.20 " = 19.7 " Match perfect. **98.0 %**	1.75 cc. N_2 at 20° and 750 mm. 0.20 gm. histidine Cl_2. **100.0 %**	0
13. Aerobic.	10 cc. 2 % histidine Cl_2. 5 cc. 2 % KNO_3. 4 cc. glycerol. 100 cc. Salt Medium 1. 81 cc. distilled water.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. **100.0 %**	0.10 cc. = 4.0 mm. 0.20 " = 8.0 " Match perfect. **40.0 %**	0.70 cc. N_2 at 23° and 753 mm. 0.0791 gm. histidine Cl_2. **39.5 %**	0
14. Anaerobic.	Same as No. 13.	0.10 cc. = 9.7 mm. 0.20 " = 19.4 " Match perfect. **97.0 %**	0.10 cc. = 9.0 mm. 0.20 " = 18.1 " Match perfect. **90.0 %**	1.6 cc. N_2 at 23° and 753 mm. 0.1807 gm. histidine Cl_2. **90.4 %**	0

Continued.

Results. Part I.

Color value of histamine fraction.*	Histidine converted into histamine (colorimetric determination).	Histidine converted into methyl imidazole (Methods B and C).	0.1 N HCl neutralized by ammonia from entire test solution.	Amount of total N as NH_3 (N_2 in KNO$_2$ ignored).	Total N as amino nitrogen with 5 cc. of test solution.	Reaction.	
						Before incubation.	After incubation.
		per cent	*cc.*	*per cent*		*pH*	*pH*
	Histamine absent.	0	41.5	66.2	1.66 cc. N_2 at 20° and 756 mm. 5.35 % total N (12.8 % histidine N).	7.35	8.3
1.0 cc. = 4.0 mm. Color too yellow; match poor.	⇔ 0.00125 gm. histamine in test solution. 0.60 % Histamine absent.	0	29.0	46.2		7.35	7.9
0.05 cc. = 12.4 mm. 0.10 " = 24.7 " Color identical with that obtained with histamine.	⇔ 0.0825 gm. histamine in test solution. **51.0 %** of histamine present.	0	0.50	1.9		7.35	>6.6
0.20 cc. = 6.4 mm. 0.40 " = 12.8 " Color identical with that obtained with histamine.	⇔ 0.01075 gm. histamine in test solution. **6.7 %** of histamine present.	0	4.5	17.1		7.35	>6.6

TABLE V

Summary of

Flask No.	Composition of completed medium; incubated 14 days at 37°.	Total color value of test solution as histidine Cl_2.* (0.20 gm. = 100 %.)	Color value of histidine fraction as histidine Cl_2.* (0.20 gm. = 100 %.)	Unchanged histidine (Van Slyke method) with 5 cc. of test solution.	Histidine converted into imidazole propionic acid (by difference)
					per cent
15. Aerobic.	10 cc. 2 % histidine Cl_2. 5 cc. 2 % KNO_3. 10 cc. 2 % NH_4Cl. 4 cc. glycerol. 100 cc. Salt Medium 1. 71 cc. distilled water.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. **100.0 %**	0.10 cc. = 4.5 mm. 0.20 " = 9.0 " Match perfect. **45.0 %**	0.85 cc. N_2 at 23° and 754 mm. 0.0961 gm. histidine Cl_2. **48.0 %**	0
16. Anaerobic.	Same as No. 15.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. **100.0 %**	0.10 cc. = 7.5 mm. 0.20 " = 15.0 " Match perfect. **75.0 %**	1.35 cc. N_2 at 20° and 753 mm. 0.1549 gm. histidine Cl_2. **77.4 %**	0
17. Aerobic.	10 cc. 2 % histidine Cl_2. 5 cc. 2 % KNO_3. 10 cc. 2 % NH_4Cl. 5 gm. glucose. 100 cc. Salt Medium 1. 70 cc. distilled water.	0.10 cc. = 9.3 mm. 0.20 " = 16.0 " Match good. **93.0 %**	0.10 cc. = 5.3 mm. 0.20 " = 8.2 " Match good. **53%**	1.06 cc. N_2 at 24° and 737 mm. 0.1168 gm. histidine Cl_2. **58.4 %**	0

* Colors matched against the (CR-MO) standard.

Concluded.

sults. Part I.

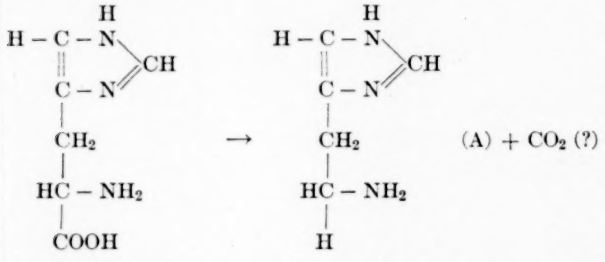
Color value of histamine fraction.*	Histidine converted into histamine (colorimetric determination).	Histidine converted into methyl imidazole (Methods B and C).	0.1 N HCl neutralized by ammonia from entire test solution.	Amount of total N as NH_3 (N_2 in KNO_2 ignored).	Total N as amino nitrogen with 5 cc. of test solution.	Reaction.	
						Before incubation.	After incubation.
		per cent	*cc.*	*per cent*		*pH*	*pH*
0.05 cc. = 12.1 mm. 0.10 " = 24.2 " Color identical with that obtained with histamine.	0.0807 gm. histamine in test solution. **50.0** % of histamine present.	0	28.4	45.3		7.35	>6.6
0.10 cc. = 7.5 mm. 0.20 " = 15.0 " Color identical with that obtained with histamine.	0.025 gm. histamine in test solution. **15.5** % of histamine present.	0	32.4	51.7		7.35	>6.6
0.10 cc. = 12.0 mm. 0.20 " = 25.3 " Color identical with that obtained with histamine.	0.0425 gm. histamine in test solution. **26.3** % of histamine present.	0	40.75	65.0		7.35	>6.6

Theoretical Considerations. Discussion of Results.

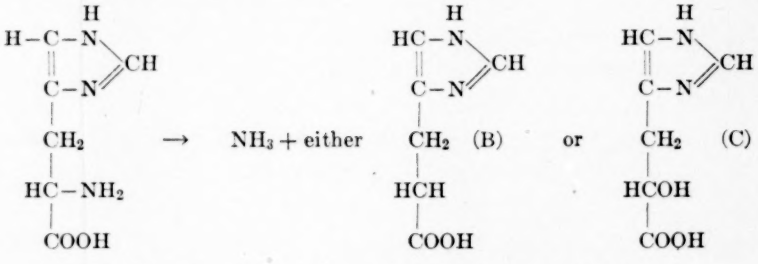
A consideration of the experiments given in Tables IV and V together with those of the other authors referred to in the introduction has led us to the following tentative theoretical deductions.

It should be possible for histidine to undergo four kinds of *primary decomposition.*

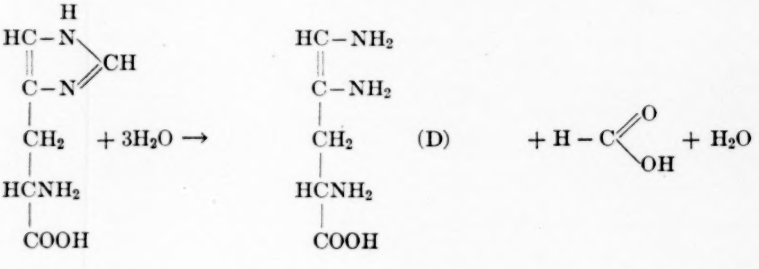
Type 1. Decarboxylation.



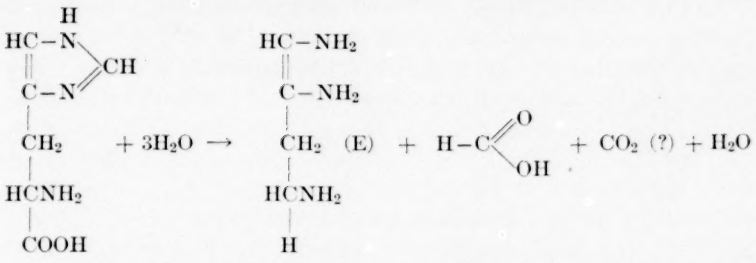
Type 2. Deamination.



Type 3. Nuclear disruption without decarboxylation.

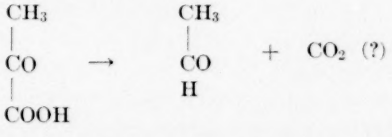


Type 4. Nuclear disruption with decarboxylation.



We have now to consider under what conditions each type of decomposition is to be expected.

Type 1.—We can see only two reasons why a microorganism would decarboxylate histidine: (1) To obtain the particular kind of carbon that is present in the carboxyl group (‡ C ‡ but more probably ± C ‡); and (2) to produce the base histamine with which excess of acidity could be neutralized more efficiently than with ammonia. Decarboxylation would lead to a lowering of the hydrogen ion concentration of the solution irrespective of whether a base was formed or not because the organic acids are usually more highly dissociated than carbonic acid. Thus the decarboxylation of pyruvic acid, for example, would lead to a reduction of the hydrogen ion concentration of the solution even though a base was not formed.



We have placed a (?) behind the CO_2 in each case because we are not certain that the carboxyl group appears immediately as CO_2, or, indeed, whether it appears in this form at all. It is possible that formic acid is first formed which later gives CO_2 by oxidation.

This type of decomposition would then be expected when another source of carbon was available and when the hydrogen ion concentration of the medium threatened to become too high for the proper development of the organism.

Type 2.—Histidine might be deaminized for three reasons.

1. The nitrogen might be removed because it was necessary for the proper development of the organism. This would be expected to occur when other available sources of nitrogen, such as ammonium salts or nitrates, were absent. In the presence of an easily available source of carbon but an inadequate source of nitrogen, the decomposition of the histidine molecule might easily cease after the nitrogen had been removed. Under these conditions imidazole lactic or propionic acids might be present unchanged at the end of the experiment.

2. The nitrogen might be removed not because it was needed, but because its removal was a necessary precursor or adjunct to the utilization of the aliphatic carbon chain. Such a decomposition might occur when other available sources of carbon were absent irrespective of the presence or absence of a nitrogen source.

3. Deamination might be resorted to as a means of lowering the hydrogen ion concentration of the medium. The equation given under Type 2 shows that the mere removal of the ammonia from the amino-acid molecule can have but little effect upon the hydrogen ion concentration of the medium because the acid radical is still present and it will neutralize the ammonia at least as completely as it did the amino group. A subsequent oxidation of the aliphatic carbon chain would destroy this acid group, however, and then the ammonia would be of service as a neutralizer of acidity. This process would be a more roundabout method for reducing the pH of a medium than direct decarboxylation. There is little doubt that this process is made use of by the body cells, and some bacteria probably proceed in the same manner.

Types 3 and 4.—A nuclear disruption, either with or without decarboxylation, is the most certain way to expose for future use all the nitrogen and carbon of the molecule. This is also a decomposition that converts a feebly basic substance into one that is strongly basic. This type of disruption might be expected if either the solution contained no other source of carbon or nitrogen than histidine or the initial pH of the medium was so markedly unfavorable to the organism that it was necessary to liberate a large amount of a base in the shortest possible time. Since a microorganism requires far more carbon than nitrogen for its growth and metabolism, a nuclear rupture would be expected to

occur even in the presence of a large excess of ammonium salts or nitrates, when the medium contained no more available source of carbon than histidine.

It is also to be expected that a substance of Type E, containing no carboxyl group, will pass into the amyl alcohol layer during the extraction process, while one of Type D, containing a carboxyl group, will remain in the alkaline aqueous layer along with the histidine.

We will now pass to a discussion of the individual cases and attempt to explain the results obtained on the basis of these theoretical deductions.

Flask 1.—In this case histidine was the only source of carbon and nitrogen. An examination of Table V shows that (1) the imidazole ring was destroyed to the extent of 98 per cent; (2) about 67 per cent of the total, not amino, nitrogen of the molecule was converted into ammonia; (3) about 13 per cent of the total nitrogen was still in the amino condition; and (4) the pH changed from 7.35 to 8.3. These results can be explained as follows.

The only source of carbon and nitrogen was histidine. A nuclear rupture would expose these elements so that they could serve as foods. Since very little of the nitrogen was required as compared to the carbon, most of it was eliminated as ammonia which lowered the hydrogen ion concentration of the solution.

Flask 2.—The composition of this medium was identical with that of No. 1 except that the oxygen concentration was almost nil. The nucleus was ruptured only to the extent of 34 per cent. The histidine that did not undergo a nuclear cleavage was untouched. About 29 per cent of the total nitrogen appeared as ammonia. The reaction of the medium changed from pH 7.35 to 7.6. The close check between the amino nitrogen and colorimetric values for histidine shows that molecules of Type D were absent. An amino nitrogen determination on the histamine fraction, which was carried out after the solution had been freed from ammonia and volatile amines, showed that 4 per cent of the total nitrogen was present in this form. This indicates that compounds of Type E were present. A nuclear rupture with decarboxylation occurred in this case which proves that *the colon bacillus has the power of decarboxylating acids, which suggests that under proper conditions it may be able to make amines from amino-acids.* All the

nitrogen introduced was accounted for. Thus 66 per cent of the histidine was recovered; 29 per cent of the nitrogen appeared as ammonia; 4 per cent was non-volatile amino nitrogen in the histamine fraction, which makes a total of 99 per cent. In this case, then, volatile amines were absent.

The explanations offered for No. 1 apply also in this case. We have found that the anaerobic differed from the aerobic experiments in quantity, not in quality. The rate of metabolism is much reduced by the oxygen deficit but the quality of the products is not affected.

Flask 3.—The results obtained in this case were almost identical with those obtained for No. 1, with the apparent exception of the ammonia value. Such an amount of ammonium chloride was originally introduced, however, that the ammonia from the entire test solution should have neutralized 36.40 cc. of 0.1 N acid. When this is subtracted from the 51.9 cc. required by experiment, 15.5 cc. of 0.1 N acid are found to have been used to neutralize the ammonia liberated from the histidine which compares very well with the 17.55 cc. required for No. 1.

The analogous results obtained in Nos. 1 and 3 can be explained by the fact that although the organism was supplied plentifully with a nitrogen source, histidine was still the only source of carbon. A nuclear rupture followed by deamination was the surest way to make this carbon available.

Flask 4.—Table V shows that 98 per cent of the histidine was recovered unchanged. The almost complete absence of metabolic activity led us to believe that the organisms had died. This proved not to be the case. This is merely another example of the necessity of oxygen for the metabolism of the colon bacillus.

Flask 5.—In this case histidine was the only source of nitrogen but glycerol was added as a source of carbon. About 80 per cent of the histidine was recovered. Here again there was a complete absence of molecules of Type D. Only 2 per cent of the histidine nitrogen was recovered as ammonia. For the first time we were confronted with a peculiar discrepancy. The total color value determination indicated that about 90 per cent of the imidazole ring was still intact. Only 80 per cent of it was proved to be histidine and the remaining 10 per cent disappeared in the extraction process. We are tempted to conclude that some heterocyclic

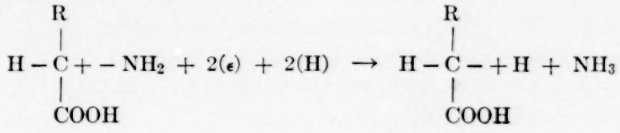
compound of undetermined constitution was present that was completely destroyed by the strong alkali used in the extraction process. This conclusion is rendered still more plausible by the subsequent experiments.

Imidazole lactic, propionic, or acetic acids were absent which, of course, means that they were either not formed under these conditions or subsequently oxidized.

In the presence of an available carbon source the histidine molecule was called upon merely as a source of nitrogen. The nitrogen was obtained partially by deamination and perhaps to a small extent by a partial rupture of the nucleus. The amount of nitrogen liberated exceeded the metabolic requirements of the organism by only 2 per cent.

Flask 6.—The composition of this medium differed from that of No. 5 only in the atmospheric oxygen content, which in this case was almost nil. Of the entire series, this is the one case in which the formation of imidazole propionic acid was indicated. Only 2 per cent of the histidine nitrogen was recovered as ammonia. Here again we were confronted with the same type of color value discrepancy that was noted with No. 5. The total color value determination indicated the presence of 100 per cent of the original imidazole ring. Only 74 per cent of this color value was accounted for as histidine and imidazole propionic acid; about 25 per cent was lost in the extraction process. This again indicates the formation of an alkali-labile heterocyclic compound. A discrepancy of this kind was obtained in only one other case (No. 8) where it amounted to 20 per cent. It seems that the labile compound is formed more easily or destroyed less easily under anaerobic conditions.

The absence of oxygen would hinder oxidation reaction and favor reductions. The formation of imidazole propionic acid from histidine is a reduction regardless of what assumptions are made as to the process.



There is no doubt that, in this case, deamination was resorted to by the colon bacillus as a means of obtaining nitrogen. To judge from the total color value determination, a nuclear disruption did not occur. It appears that this organism is readily capable of rupturing the imidazole ring only in the presence of oxygen.

Flask 7.—This medium contained both an easily available source of carbon and nitrogen. Table V shows that: (1) this is the first member of the series that was distinctly acid after incubation; (2) 46.4 per cent of the histidine was converted into histamine; (3) unchanged histidine was the only other product; therefore (4) a marked nuclear rupture did not occur; and (5) about 50 per cent of the ammonia that was originally introduced was consumed by the bacteria.

The histidine in this medium was unnecessary as a food constituent because the organisms grew perfectly with just the glycerol and ammonium salt to serve as carbon and nitrogen sources. Under these conditions neither a nuclear disruption nor deamination could be expected unless a nuclear rupture was resorted to as a means of neutralizing excess acidity. Decarboxylation occurred to a very marked extent—46.4 per cent—which proves that the colon bacillus has this power. We concluded (see Theoretical Considerations) that the organism would decarboxylate histidine either to get the carboxyl carbon or to manufacture the strong base histamine. This experiment leaves us largely in the dark as to the purpose of the decarboxylation. The fact that histamine was never found, in our experiments, unless the medium had become distinctly acid, suggests strongly that the base was formed merely to lower the hydrogen ion concentration of the solution. In Part III of this paper, proof that this is actually the case is presented.

Flask 8.—This was the anaerobic equivalent of No. 7. Table V shows that (1) the medium was distinctly acid after incubation; (2) 13.7 per cent of the histidine was converted into histamine as compared to 46.4 per cent in No. 7; (3) unchanged histidine was the only other recognizable product so that here again there is the indication that an alkali-labile heterocyclic compound was formed to the extent of 20 per cent; (4) there was no evidence of a nuclear disruption; (5) about 40 per cent of the ammonia that was originally introduced was removed from the solution by the bacteria.

The discussion given for No. 7 applies also to this case. The oxygen deficit decreased the rate of metabolism.

Flasks 9 to 16 inclusive.—The remaining experiments were duplicates of those already described except that potassium nitrate was present in all the media. We introduced the nitrate to see whether it would have any effect upon the production of histamine. Potassium nitrate might be expected to function in one or more of the following ways: (1) as a source of potassium or nitrogen; (2) as a source of alkali and ammonia, both of which might be used for the purpose of neutralizing excess of acidity;

$$KNO_3 + 8(H) \rightarrow KOH + NH_3 + 2H_2O$$

(3) as a source of oxygen;

$$KNO_3 + 2(H) + H_2O \rightarrow KOH + NH_3 + 3(O)$$

In this case the ammonia need not, of course, be liberated as such, but might be used for synthetic purposes.

The results obtained in Flasks 13 and 14 prove that KNO_3 is a very efficient source of nitrogen for the colon bacillus. These results were fairly close duplicates of those obtained in Flasks 7 and 8 in which ammonium chloride was present instead of potassium nitrate. A slightly larger percentage of histamine was always formed in the presence of the nitrate than in the presence of the ammonium salt. Thus Flasks 15 and 16 contained more histamine than Flasks 7 and 8 respectively.

In Flask 14, some of the KNO_3 must have been converted into ammonia. In this case 97 per cent of the total nitrogen was present either as histidine or histamine. If all the remaining 3 per cent of nitrogen had been converted into ammonia, the entire test solution would have contained enough of it to neutralize only 0.80 cc. of the 0.10 N acid. Since 4.5 cc. of the acid were actually neutralized by ammonia arising from this solution, we must conclude that enough NH_3 to neutralize 3.7 cc. of the acid must have been formed from the KNO_3 which shows that at least 37 per cent of the nitrate was converted into ammonia.

For the colon bacillus, potassium nitrate seems to serve chiefly as a source of nitrogen. With this in mind the results obtained in Experiments 9, 10, 11, and 12 can be readily understood since they agree perfectly with the theoretical stipulations.

Flask 17.—The nutritive conditions were identical, in this case, to those of No. 15 except that glucose was used as a carbon source in place of glycerol. The results obtained may be summarized as follows: (1) the medium was distinctly acid after incubation; (2) 26.3 per cent of the histidine was converted into histamine as compared to 46.4 per cent in No. 7; (3) about 58 per cent of the histidine was unchanged; (4) a nuclear disruption had occurred to the extent of about 15 per cent; and (5) ammonia was generated by the bacteria either from the histidine or from the potassium nitrate. In either case, this ammonia would help to reduce the hydrogen ion concentration of the medium.

Since the nutritive conditions of this experiment were identical with those of No. 15, except for the carbon source, the discovery that 26 per cent of the histidine was converted into histamine would be no surprise if one had not read the literature. The statement is repeatedly made that glucose prevents the decarboxylation of amino-acids by bacteria. Thus Mellanby and Twort,[7] Berthelot and Bertrand,[8] and Jones[11] claim that histamine is not produced in the presence of glucose. The experimental conditions employed by these observers were different from our own and this may account for the difference in results. As will be shown in Parts II and III, such factors as time of incubation and the initial concentration of disodium acid phosphate have a very decided influence upon the formation of histamine from histidine.

There seems to have been a tendency, in the past, to confuse the sparing action of carbohydrates for proteins and the effect of carbohydrates on the decarboxylation of amino-acids. That a body cell or a microorganism will obtain its carbon requirements from carbohydrates by preference to amino-acids or proteins has been proved beyond doubt. Our own experiments show quite conclusively that this principle is correct. Thus in Nos. 1, 3, 9, and 11 where histidine was the only source of carbon, a complete decomposition of the molecule occurred, the histidine being used as a carbon source. When glycerol was added, the histidine remained untouched except as it was called upon to supply nitrogen or to neutralize acidity. The idea that the carbohydrate would also prevent decarboxylation must have arisen because it

[11] Jones, H. M., *J. Infect. Dis.*, 1918, xxii, 125.

was thought that the carboxyl carbon served as a carbon source. If this were actually the case it would be easy to see why the addition of an easily available source of carbon would render decarboxylation unnecessary. The facts are, however, that decarboxylation with amine production occurs only in the presence of an outside source of carbon and that this can be prevented by neutralizing the excess of acidity (see Part III).

These statements apply, of course, only to the colon bacillus. We shall report, at a later date, experiments conducted with other microorganisms and, until these experiments have been completed, a generalization is not admissible.

Part II. The Effect of Duration of Incubation upon Conversion of Histidine into Histamine.

The solutions employed, the mode of procedure, and the methods of analysis were identical with those described in Part I. These experiments were duplicates of No. 15 of Part I except that the time of incubation varied from 2 to 40 days.

The composition of the salt medium did not differ materially from that of Experiment 15. The concentration of the sodium chloride was slightly reduced and the potassium chloride was eliminated. The ammonium chloride and potassium nitrate were incorporated into the salt medium.

Composition of Salt Medium 2.— 4.00 gm. of ammonium chloride.
2.00 " " potassium nitrate.
8.00 " " " dihydrogen phosphate.
16.00 " " sodium chloride.
0.40 " " " sulfate.
8.00 " " " bicarbonate.
0.20 " " calcium chloride.

These amounts were dissolved in water and diluted to exactly 2,000 cc.

Composition of Completed Media.—10 cc. of 2 per cent histidine dichloride.
4 " " glycerol.
100 " " Salt Medium 2.
86 " " distilled water.

These amounts were mixed in a 300 cc. Pyrex Florence flask. The salt medium was added from a 100 cc. normal pipette.

The results are given in Table VI.

Flask No.	Composition of completed medium.	Time of incubation.	Total color value of test solution as histidine Cl_2.* (0.20 gm. = 100 %.)	Color value of histidine fraction as histidine Cl_2.* (0.20 gm. = 100 %.)	Unchanged histidine (Van Slyke method) with 5 cc. of test solution.	Histidine converted into imidazole propionic acid
		days				per cent
1	10 cc. 2 % histidine Cl_2. 4 cc. glycerol. 100 cc. Salt Medium 2. 86 cc. distilled water.	2	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. **100.0** %	0.10 cc. = 9.2 mm. 0.20 " = 18.3 " Match perfect. **92.0** %	1.70 cc. N_2 at 22° and 746 mm. 0.191 gm. histidine Cl_2. **95.0** %	0
2	Same as No. 1.	5	0.10 cc. = 10.0 mm. 0.20 " = 20.1 " Match perfect. **100.0** %	0.10 cc. = 8.7 mm. 0.20 " = 17.5 " Match perfect. **87.0** %	1.52 cc. N_2 at 22° and 746 mm. 0.1708 gm. histidine Cl_2. **85.5** %	0
3	" " " 1.	10	0.10 cc. = 10.1 mm. 0.20 " = 20.2 " Match perfect. **101.0** %	0.10 cc. = 7.4 mm. 0.20 " = 14.8 " Match perfect. **74.0** %	1.38 cc. N_2 at 24° and 737 mm. 0.152 gm. histidine Cl_2. **76.0** %	0
4	" " " 1.	14†	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. **100.0** %	0.10 cc. = 4.5 mm. 0.20 " = 9.0 " Match perfect. **45.0** %	0.85 cc. N_2 at 23° and 754 mm. 0.0961 gm. histidine Cl_2. **48.0** %	0
5	" " " 1.	20	0.10 cc. = 11.4 mm. 0.20 " = 23.0 " Color more like that of histamine than histidine. **114.0** %	0.10 cc. = 1.4 mm. 0.50 " = 7.0 " Match perfect. **14.0** %	0.33 cc. N_2 at 24° and 750 mm. 0.037 gm. histidine Cl_2. **18.5** %	0

Results. Part II.

Color value of histamine fraction.*	Histidine converted into histamine (colorimetric determination).	Histidine converted into methyl imidazole (Methods B and C).	Amount of 0.1 N HCl neutralized by ammonia from entire test solution.	Total N as NH_2 (N_2 in KNO$_3$ ignored).	Per cent of histidine that had been converted into histamine (Van Slyke method). 5 cc. of the test solution gave.	Reaction.	
						Before incubation	After incubation.
		per cent	*cc.*	*per cent*		*pH*	*pH*
1.0 cc. = 4.0 mm. Color greenish yellow; match poor.	Histamine absent.	0	39.75	63.4		7.35	6.8
0.40 cc. = 6.7 mm. Match good.	0.0053 gm. histamine in test solution. **3.3** % of histamine present.	0	39.75	63.4		7.35	Slightly >6.6
0.10 cc. = 11.8 mm. 0.20 " = 23.5 " Match perfect; identical with that obtained with histamine.	0.039375 gm. histamine in test solution. **24.4** % of histamine present.	0	38.25	61.0		7.35	>6.6
0.05 cc. = 12.1 mm. 0.10 " = 24.2 " Match perfect; identical with that obtained with histamine.	0.0807 gm. histamine in test solution. **50.0** % of histamine present.	0	28.4	45.3		7.35	>6.6
0.025 cc. = 9.4 mm. 0.05 " = 18.8 " Match perfect; identical with that obtained with histamine.	0.12625 gm. histamine in test solution. **78.0** % of histamine present.	0	31.5	50.2	1.37 cc. N_2 at 24° and 747 mm. 0.1237 gm. histamine Cl_2. **77.0** %	7.35	>6.6

TABLE VI

Summary of

Flask No.	Composition of completed medium.	Time of incubation.	Total color value of test solution as histidine Cl_2.* (0.20 gm. = 100 %.)	Color value of histidine fraction as histidine Cl_2.* (0.20 gm. = 100 %.)	Unchanged histidine (Van Slyke method) with 5 cc. of test solution.	Histidine converted into imidazole propionic acid (by difference)
		days				*per cent*
6	Same as No. 1.	30	0.10 cc. = 11.3 mm. 0.20 " = 22.5 " Color more like that of histamine than histidine. **113.0** %	0.50 cc. = 4.0 mm. Match good. **8.0** %	0.33 cc. N_2 at 24° and 750 mm. 0.037 gm. histidine Cl_2. **18.5** %	0
7	" " " 1.	40	0.10 cc. = 11.3 mm. 0.20 " = 22.6 " Color more like that of histamine than histidine. **113.0** %	0.50 cc. = 4.0 mm. Match good. **8.0** %	0.33 cc. N_2 at 21° and 751 mm. 0.0377 gm. histidine Cl_2. **18.8** %	0

* Colors matched against the (CR–MO) standard.

† Copied from Table V, Flask No. 15.

Concluded.

Results. Part II.

Color value of histamine fraction.*	Histidine converted into histamine (colorimetric determination).	Histidine converted into methyl imidazole (Methods B and C).	Amount of 0.1 N HCl neutralized by ammonia from entire test solution.	Total N as NH_3 (N_2 in KNO_2 ignored).	Per cent of histidine that had been converted into histamine (Van Slyke method). 5 cc. of the test solution gave.	Reaction.	
						Before incubation.	After incubation.
		per cent	*cc.*	*per cent*		*pH*	*pH*
0.025 cc. = 9.9 mm. 0.05 " = 19.8 " Match perfect; identical with that obtained with histamine.	0.1325 gm. histamine in test solution. **82.0** % of histamine present.	0	25.5	40.7	1.40 cc. N_2 at 21° and 752 mm. 0.1291 gm. histamine Cl_2. **80.0** %	7.35	>6.6
0.025 cc. = 10.1 mm. 0.05 " = 20.2 " Match perfect; identical with that obtained with histamine.	0.135 gm. histamine in test solution. **83.5** % of histamine present.	0	20.0	32.0	1.53 cc. N_2 at 24° and 752 mm. 0.139 gm. histamine Cl_2. **86.0** %	7.35	>6.6

Although the table is largely self explanatory, the following facts call for special emphasis.

The organisms multiplied abundantly during the first 2 days. Some acid was produced but not enough to counteract the neutralizing effect of the $KNaHPO_4$. Ammonia was formed either from the nitrate or from the histidine. *Histamine was absent.*

During the next 3 days enough acid had been formed to counteract entirely the neutralizing ability of the phosphate and the generated ammonia. *During this period 3.3 per cent of the histidine was converted into histamine.*

During the next 5 days decarboxylation proceeded rapidly so that a total of 24.4 per cent of the histidine was converted into histamine. The concentration of ammonia began to drop as it was used to supply the nitrogen requirements of the organism.

During the next 10 days there was an enormous increase in the concentration of the histamine so that 78 per cent of the histidine had been decarboxylated during the entire 20 day period.

The next 20 days produced a slight but steady increase in the histamine concentration and a marked decrease in the ammonia concentration. During this period the process of decarboxylation, as a means of neutralizing acidity, seems to have given place to the far more efficient process of nuclear disruption; for although the color value determination indicated the presence of only 8 per cent of histidine, the amino nitrogen determination showed that the equivalent of 18 per cent of histidine was present. This shows that about 3 per cent of the histidine had undergone a nuclear rupture with the formation of a compound of Type D (see p. 564). The absence of appreciable quantities of compounds of Type E was proved by the close check between the colorimetric and the amino nitrogen determinations on the histamine fraction. A rupture of the imidazole ring in the histamine molecule did not occur during these 40 days of incubation.

Finally, we wish again to call attention to the fact that histamine appeared only in the media that had become distinctly acid during the incubation period.

Part III. Histamine May Be Formed by the Colon Bacillus to Lower the Hydrogen Ion Concentration of the Medium.

The solutions employed, the mode of procedure, and the methods of analysis were identical with those described in Part I. The composition of the salt medium was changed so that the glycerol became a part of it, which made it unnecessary to add this substance separately. We repeat the composition of this complete medium so that it may be referred to conveniently in our later reports.

Composition of Nutritive Medium 3.— 4.00 gm. of ammonium chloride.
2.00 " " potassium nitrate.
8.00 " " " dihydrogen phosphate.
16.00 " " sodium chloride.
0.40 " " anhydrous sodium sulfate.
8.00 " " sodium bicarbonate.
0.20 " " anhydrous calcium chloride.
80.00 cc " glycerol.

These amounts were dissolved in water and diluted to exactly 2,000 cc.

Composition of the Completed Media.—

No. 1. 10 cc. of 2 per cent histidine dichloride.
100 " " Nutritive Medium 3.
90 " " distilled water.
2.5 gm. of $Na_2HPO_4.12\ H_2O$.

No. 2. 10 cc. of 2 per cent histidine dichloride.
100 " " Nutritive Medium 3.
90 " " distilled water.
3.8 gm. of $Na_2HPO_4.12\ H_2O$.

No. 3. 10 cc. of 2 per cent histidine dichloride.
100 " " Nutritive Medium 3.
90 " " distilled water.
0.50 gm. of sodium formate.

No. 4. 10 cc. of 2 per cent histidine dichloride.
100 " " Nutritive Medium 3.
90 " " distilled water.
1 " " 2.5 per cent filtered litmus solution.

DISCUSSION.

The four flasks were incubated at 37° for 14 days. Flask 4 was equipped with a two-hole rubber stopper into which were inserted a dropping funnel and a glass tube that served as an air vent. During the 2 week period, 14 cc. of normal sodium hydroxide and 2 cc. of sterile 2.5 per cent litmus solution were added to the contents of the flask through the funnel. The reaction of the medium was slightly toward the acid side of neutrality all the time and it became distinctly acid a few times. The addition of the litmus solution was necessary because the bacterial action discharged the color of the mixture entirely.

An abundant growth was obtained in all the flasks. The bacteria were alive at the end of the experiment. None of the media was contaminated.

The litmus that remained in Flask 4 at the close of the experiment did not interfere with the colorimetric determinations because practically all of it appeared in the form of a coagulum when the filtered liquid was evaporated with 1 cc. of 95 per cent sulfuric acid.

The results are given in Table VII.

The following facts call for special emphasis and interpretation.

The media employed in these experiments were identical with those of Part II of this paper, except that various sources of alkali were added. In Experiment 4 of Part II, the time of incubation was 14 days which makes it analogous to the experiments now under consideration. In this time interval 50 per cent of the histidine had been converted into histamine.

In Experiment 1 of Part III, where 2.5 gm. of hydrated basic sodium phosphate were added to control the pH of the medium, only 10 per cent of the histidine had been converted into histamine. When, in Experiment 2, the concentration of the phosphate was increased so that a total of 3.8 gm. had been added, only 4 per cent of the histidine was converted into histamine. Some free acid had accumulated in both cases which shows that not enough basic phosphate had been added to neutralize entirely the accumulating acid.

From these experiments one would be tempted to conclude that histamine was produced only after the neutralizing effect of the basic phosphate had been overcome by the accumulating acid.

The initial addition of more phosphate would keep the solution neutral for a longer time; hence would delay the production of histamine. This would seem to indicate strongly that the base histamine was produced from histidine to reduce the pH of the solution. When basic phosphate was added, the production of the amine was rendered unnecessary for a time because it, the phosphate, prevented a rise in the pH of the medium.

It might be claimed, however, that in some way the phosphate inhibited the decarboxylase activity of the microorganism; therefore, Experiment 4 was carried out. In this case sodium hydroxide was added from time to time to keep the solution as nearly neutral as possible. Although a luxuriant growth was obtained, only 1 per cent of the histidine was decarboxylated under these conditions. There can be no doubt, then, that in this case the neutralization of the medium prevented, or, as we might say, rendered unnecessary, the production of histamine.

Experiment 3 was originally carried out to see if the addition of carboxyl carbon in an easily available form would prevent the decarboxylation of histidine. The addition of formic acid in any but very small concentrations was not feasible because it would raise the pH of the medium to such an extent that the organisms would be unable to multiply properly. Sodium formate was, therefore, resorted to. We soon saw, however, that this experiment would not prove the point for the following reasons.

Omelianski[12] has shown that sodium formate is rapidly decomposed by *Bacterium formicum* according to the equation

$$2(HCOONa + H_2O) \rightarrow Na_2CO_3 + H_2O + CO_2 + 2H_2$$

Pakes and Jollyman,[13] and Karczag and Schiff[14] have shown that formic acid is similarly decomposed by the colon bacillus. Since this decomposition is one that proceeds readily and rapidly, sodium formate is really an excellent source of alkali since it yields sodium carbonate when it is acted upon by bacteria. Instead of decarboxylating histidine to obtain the base histamine, sodium formate was decarboxylated which gave rise to the very

[12] Omelianski, W., *Centr. Bacteriol., 2te Abt.*, 1904, xi, 177, 256, 317.

[13] Pakes, W. C. C., and Jollyman, W. H., *J. Chem. Soc.*, 1901, lxxix, 322, 386, 459.

[14] Karczag, L., and Schiff, E., *Biochem. Z.*, 1915, lxx, 329.

Flask No.	Composition of completed medium; incubated for 14 days at 37°.	Total color value of test solution as histidine Cl_2.* (0.20 gm. = 100 %.)	Color value of histidine fraction as histidine Cl_2.* (0.20 gm. = 100 %.)	Unchanged histidine (Van Slyke method) with 5 cc. of test solution.	Histidine converted into imidazole propionic acid
					Per cent
1	10 cc. 2 % histidine Cl_2. 100 cc. Nutritive Medium 3. 90 cc. distilled water. 2.5 gm. Na_2HPO_4. 12 H_2O.	0.10 cc. = 9.3 mm. 0.20 " = 18.5 " Match perfect. **93.0** %	0.10 cc. = 7.6 mm. 0.20 " = 15.2 " Match perfect. **76.0** %	1.4 cc. N_2 at 22° and 745 mm. 0.1575 gm. histidine Cl_2. **78.8** %	0
2	10 cc. 2 % histidine Cl_2. 100 cc. Nutritive Medium 3. 90 cc. distilled water. 3.8 gm. Na_2HPO_4. 12 H_2O.	0.10 cc. = 8.6 mm. 0.20 " = 17.2 " Match perfect. **86.0** %	0.10 cc. = 8.3 mm. 0.20 " = 16.7 " Match perfect. **83.0** %	1.5 cc. N_2 at 26° and 754 mm. 0.167 gm. histidine Cl_2. **83.5** %	0
3	10 cc. 2 % histidine Cl_2. 100 cc. Nutritive Medium 3. 90 cc. distilled water. 0.5 gm. sodium formate.	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. **100.0** %	0.10 cc. = 9.2 mm. 0.20 " = 18.2 " Match perfect. **92.0** %	1.73 cc. N_2 at 27° and 747 mm. 0.1895 gm. histidine Cl_2. **95.0** %	0
4	10 cc. 2 % histidine Cl_2. 100 cc. Nutritive Medium 3. 90 cc. distilled water. 1 cc. 2.5 % filtered litmus solution.†	0.10 cc. = 10.0 mm. 0.20 " = 20.0 " Match perfect. **100.0** %	0.10 cc. = 9.8 mm. 0.20 " = 19.7 " Match perfect. **98.0** %	1.77 cc. N_2 at 26° and 754 mm. 0.1974 gm. histidine Cl_2. **98.7** %	0

* Colors matched against the (CR–MO) standard.

† See special record, page 580.

‡ This is probably high by 0.6 per cent due to admixture with histidine.

Results. Part III.

Color value of histamine fraction.*	Histidine converted into histamine (colorimetric determination).	Histidine converted into methyl imidazole (Methods B and C).	Amount of 0.1 N HCl neutralized by ammonia from entire test solution.	Total N as NH_3 (N_2 in KNO_2 ignored).	Histidine converted into histamine (Van Slyke method) with 5 cc. of test solution.	Reaction.	
						Before incubation.	After incubation.
		per cent	*cc.*	*per cent*		*pH*	*pH*
0.10 cc. = 5.0 mm. 0.50 " = 25.1 " Match perfect; identical with that obtained with histamine.	0.0165 gm. histamine in test solution. **10.2** % of histamine present.	0	23.0	36.7	0.22 cc. N_2 at 22° and 750 mm. 0.02011 gm. histamine Cl_2. **12.5** %	7.5	>6.6
0.50 cc. = 10.2 mm. 1.0 " = 20.2 " Match perfect; identical with that obtained with histamine.	0.0068 gm. histamine in test solution. **4.2** % of histamine present.	0	23.5	37.5	0.06 cc. N_2 at 30° and 752 mm. 0.00527 gm. histamine Cl_2. **3.25** %	7.7	Slighlty >6.6
1.0 cc. = 9.7 mm. Match good for histamine.	0.00325 gm. histamine in test solution. **2.0** % of histamine present.	0	34.7	55.3		7.4	6.6
1.0 cc. = 5.7 mm. Match good; more like histidine than histamine.	0.00175 gm. histamine in test solution. **1.08** %‡ of histamine present.	0	18.5	29.5	0.02 cc. N_2 at 30° and 752 mm. 0.001758 gm. histamine Cl_2. **1.09** %	Medium kept approximately neutral to litmus by addition of N NaOH.†	

much more efficient acid neutralizer sodium carbonate. In this case then, only 2 per cent of the histidine was converted into histamine.

SUMMARY.

1. When the colon bacillus is allowed to metabolize histidine, either alone or in the presence of nitrates or ammonium salts, histamine is not formed.

2. In a medium containing histidine and glycerol, but no nitrates or ammonium salts, histamine is not formed. In this case imidazole propionic acid appears to be formed; but only when the bacillus is forced to grow anaerobically.

3. In a medium containing histidine, glycerol, or glucose and a source of nitrogen, either KNO_3, NH_4Cl, or both, about 50 per cent of the histidine is converted into histamine in the course of 2 weeks when oxygen is present. In the absence of atmospheric oxygen, this and all the other metabolic activities of the bacillus are greatly reduced, probably because the colon bacillus is an aerobic organism by preference.

4. The production of histamine is always coincident with the production of a medium that is distinctly acid. We believe that the histamine is formed by the bacillus to neutralize the excess of acidity that is simultaneously produced from the glycerol.

5. Contrary to the statement sometimes given in text-books and in the literature that carbohydrates prevent the formation of histamine from histidine, we have found that histamine is never formed except in the presence of an easily available source of carbon such as glycerol or glucose.

STUDIES ON PROTEINOGENOUS AMINES.

V. THE PREPARATION OF *P*-HYDROXYPHENYLETHYLAMINE HYDROCHLORIDE (TYRAMINE HYDROCHLORIDE).

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(Received for publication, July 14, 1919.)

The synthesis of *p*-hydroxyphenylethylamine reported herewith is based on the synthesis of *p*-hydroxybenzylcyanide reported by Pschorr, Wolfes, and Buckow[1] and on its reduction as reported by Barger.[2] Benzylcyanide is nitrated, *p*-aminobenzylcyanide obtained by reduction of the nitro compound, and the *p*-hydroxybenzylcyanide, obtained with the aid of nitrous acid, is then reduced with sodium and alcohol. The improvements that we have to suggest on the first three steps are largely mechanical. The preparation and purification of the final product, *p*-hydroxyphenylethylamine, was carried out according to the scheme previously developed for histamine[3] which is rather more efficient than the method reported by Barger.

The nitration of benzylcyanide offers no difficulties when the method of Pschorr, Wolfes, and Buckow[1] is carefully followed. It is necessary to purify the product thoroughly by recrystallization from alcohol before proceeding to the next preparation. An impure product gives rise to a highly colored amino compound on reduction which is hard to purify.

Preparation of p-Aminobenzylcyanide.

In the preparation of *p*-aminobenzylcyanide, the following further suggestions may be of value.

[1] Pschorr, R., Wolfes, O., and Buckow, W., *Ber. chem. Ges.*, 1900, xxxiii, 170.

[2] Barger, G., *J. Chem. Soc.*, 1909, xcv, 1127.

[3] Koessler, K. K., and Hanke, M. T., *J. Am. Chem. Soc.*, 1918, xl, 1722.

After the reduction is complete and the alcohol has been removed by distillation *in vacuo* at 40°[1] the mixture is cooled in an ice bath, covered with a layer of 250 cc. of ether, and treated with a solution of 70 gm. of sodium hydroxide in 150 cc. of water, the alkali being added very slowly so that the temperature of the mixture does not rise above 20°. After all the alkali has been added, two sharply defined layers are obtained, the lower being slightly grey due to precipitated tin, the upper being very pale yellow. The ether layer is decanted as completely as possible, and the aqueous alkaline layer reextracted four times with ether, 200 cc. being used for each extraction. The combined ether extracts, which should be nearly colorless, are dried rapidly over anhydrous sodium carbonate and the ether removed by distillation, at first at ordinary pressure and then *in vacuo*. An entirely crystalline, pale yellow solid was usually obtained, with a melting point of 44–46° and whose weight was from 95 to 98 per cent of that demanded by the theory. If a dark-colored product is obtained, which will never be the case when all the precautions given above are observed, this can be readily purified by a vacuum distillation. This gives a pale green liquid, whose boiling point is 198° at a pressure of 10 mm., which solidifies readily in the ice box to a pale greenish white solid that soon becomes almost colorless and melts at 45–46°. The product can be kept indefinitely in a tightly stoppered bottle at ice box temperature. At room temperature it gradually becomes brown and sticky.

Preparation of p-Hydroxybenzylcyanide.

Although the description given by Pschorr, Wolfes, and Buckow of the preparation of *p*-hydroxybenzylcyanide leads one to believe that they had the details carefully worked out, they do not report them completely enough to make an exact repetition possible. We take the liberty, therefore, to report the details of our experiments.

A solution of 20 cc. of 95 per cent sulfuric acid in 200 cc. of water is heated to boiling over an asbestos gauze in a 1,000 cc. Florence flask. 6.6 gm. of *p*-aminobenzylcyanide—0.05 mol—is then added to the gently boiling solution. The locally precipitated sulfate of the base dissolves rapidly in the hot solution. The

flame is turned low so that the liquid is kept just below the boiling point. The flask is then equipped with a three-hole rubber stopper into which are inserted a thermometer and a dropping funnel, both of which extend to within a few mm. of the bottom of the flask. A solution of 4 gm. of 90 per cent sodium nitrite in 40 cc. of water is allowed to flow steadily under the acid liquid in the course of 15 minutes. The temperature of the liquid should remain between 95–100°. The liquid appears to boil due to the steady evolution of nitrogen which should contain little or no nitrous anhydride. 50 cc. of water are then added to the reaction liquid, and the contents of the flask heated to boiling.

The solution so obtained, because of its high acid content, is free from the purple dye that is always formed if too little acid is present. A very little dark brown tar is formed. To remove this, the *hot* liquid is treated with about 5 gm of animal charcoal and filtered into a 1,000 cc. flask. The yellow filtrate is cooled in tap water. A colorless oil is deposited which is *p*-hydroxybenzylcyanide. On long standing, or quickly after seeding, the oil solidifies, so a primary crop of crystals could be obtained in this way. It is not advisable to separate this material at this time, however, because an ether extraction of the acid liquid is necessary under any condition. The cold, cloudy, yellow, acid liquid is, therefore, extracted four times in the flask with ether, using about 200 cc. of ether for each extraction. (The first extract contains practically all the *p*-hydroxybenzylcyanide.) The pale yellow ether solution is then extracted twice with 25 cc. of a saturated aqueous solution of bicarbonate of soda, and once with 25 cc. of water. The bicarbonate solution removes all the *p*-hydroxyphenylacetic acid and nearly all the coloring matter. The ether is then removed by distillation, at first under ordinary pressure and then *in vacuo*. There is thus obtained 5.5 gm. of nearly pure substance in the form of a pale yellow crystal cake. This is 83 per cent of that required by theory.

The substance so obtained can be further purified by distillation *in vacuo*—it boils at 210° at 10 mm. pressure—or it can be recrystallized from boiling water. The crystal cake is dissolved in 10 parts of boiling water, the solution clarified with a little animal charcoal after adding a few drops of 37 per cent hydrochloric acid, and filtered. On cooling and seeding the filtrate

deposits a colorless oil which rapidly becomes entirely crystalline. The white crystals weigh 75 per cent of the starting material and melt at 67–71°. To obtain a second crop it is advisable to extract the mother liquor with ether and extract the ether with bicarbonate solution as above.

Preparation of p-Hydroxyphenylethylamine Hydrochloride (Tyramine Hydrochloride).

Barger prepared the free base of the above substance by a process that was laborious and time-consuming. He made no attempt to isolate the by-products. The method outlined below gives all the products that are formed in the reaction in a fairly high state of purity, the tyramine being obtained in its most desirable form, the hydrochloride.

5 gm. of *p*-hydroxybenzylcyanide, melting point 168–171°, were treated with 50 cc. of absolute alcohol in a 500 cc. round bottomed flask. The flask was suspended from a Liebig condenser by means of a rubber stopper, and the alcohol heated to boiling by means of a small flame which impinged against an asbestos gauze. A ½ inch air space between the gauze and the bottom of the flask prevented superheating and charring of the sodium ethylate which precipitated in the course of the reaction. 10 gm. of metallic sodium, about two equivalents, were rapidly added through the condenser in small pieces, the time of addition being 5 minutes. After boiling for ½ hour, 20 cc. of absolute alcohol were added through the condenser to facilitate the solution of the sodium, and to dissolve the precipitated sodium ethylate. It was necessary to add 20 cc. more of absolute alcohol after 45 minutes of total heating, to dissolve all the sodium.

After the metal had completely dissolved, the mixture was allowed to cool somewhat. 100 cc. of water were then added to the warm mixture. The resulting clear, yellow solution was freed from alcohol, ammonia, and some water by distillation *in vacuo* to a volume of about 50 cc. Enough concentrated hydrochloric acid was then added to give a strongly acid reaction to litmus, the flask and contents being cooled in tap water while the acid was being added. The resulting pale yellow solution was transferred with a little water to a graduated separatory funnel.

Its final volume was about 150 cc. A small amount of oil, *p*-cresol, floated on the aqueous liquid.

Extraction of p-Cresol and p-Hydroxyphenylacetic Acid.

The solution was freed from the above two compounds by extracting it five times with ether, using 200 cc. of ether for each extraction. The further treatment of this ether extract will be discussed below.

Extraction of Tyramine.—The liquid so obtained was rendered strongly alkaline by the addition of anhydrous sodium carbonate and extracted exhaustively with amyl alcohol using 150 cc. of the alcohol for each extraction. From seven to ten extractions were necessary to remove the tyramine completely. The aqueous layer gave only a pale pink Pauly reaction when the extraction was complete. It was discarded.

The combined amyl alcohol extracts were dried over anhydrous sodium carbonate, filtered from the carbonate using a Buchner funnel, and the clear, pale brown filtrate was extracted three times with 1.0 N HCl and seven times with water using 100 cc. for each extraction.

The combined aqueous acid extracts were freed from water and hydrochloric acid by distillation *in vacuo* at 50°. A voluminous, pale yellow solid was left in the flask which weighed 3.8 gm. and which was nearly pure tyramine hydrochloride. This is 58 per cent of that required by theory.

The solid obtained from three experiments, 11.1 gm., was treated with 5 cc. of concentrated hydrochloric acid and 100 cc. of absolute alcohol. By heating on the water bath practically all the solid was brought into solution. The liquid was filtered from a slight residue of sodium chloride. On cooling the filtrate, tyramine hydrochloride crystallized out in the form of fluffy, glistening needles. The mixture was filtered with suction and the crystals were washed freely with absolute alcohol. Dried *in vacuo* for 48 hours over sulfuric acid, the pure white solid weighed 9.1 gm. and had the following properties.

1. It melted to a clear brown liquid at 280° (Corrected).
2. It was entirely free from ammonium chloride.
3. It gave an intense orange red color reaction with paraphenyldiazonium sulfonate.

4. It left a residue on ignition at dull red heat of 1.86 per cent; *i.e.*, 0.3000 gm. ignited in a platinum crucible left a residue of 0.0056 gm., which proved to be sodium chloride. This would suggest that the product was 98.14 per cent pure.

5. A Van Slyke amino nitrogen determination gave the following results. 0.0100 gm. of substance gave 1.48 cc. of nitrogen gas at 28° and 749 mm. If the material was 98.14 per cent pure, the actual weight of pure tyramine hydrochloride used was 0.009814 gm.

	Calculated.	Found.
Per cent amino nitrogen	8.06	8.11

6. A chlorine determination gave the following results. 0.2000 gm. of solid took 11.87 cc. of 0.1 N $AgNO_3$. If the solid is considered to be 98.14 per cent tyramine hydrochloride and 1.86 per cent sodium chloride, 11.93 cc. should have been used.

7. A solution containing 0.002 gm. of substance was injected into the femoral vein of a dog, weighing 4.7 kilos, that had been previously anesthetized and prepared so that a blood pressure tracing could be obtained. The tracing obtained, Fig. 1, showed that the typical rise in blood pressure had been produced by the substance.

This series of seven tests proves conclusively that the substance was *p*-hydroxyphenylethylamine. Its purity was 98.14 per cent. The remaining 1.86 per cent consisted entirely of sodium chloride.

Separation of p-Cresol from p-Hydroxyphenylacetic Acid.—The combined ether extracts from five experiments were concentrated to a volume of about 1,000 cc. The ether was extracted five times with a 10 per cent sodium carbonate solution using 40 cc. for each extraction. The combined carbonate solutions were then extracted five times with ether using 100 cc. for each extraction. This divided the material into two fractions; the ether fraction which contained all the *p*-cresol and was free from *p*-hydroxyphenylacetic acid, and the alkaline aqueous fraction which was free from cresol but contained all the *p*-hydroxyphenylacetic acid.

Separation of Pure p-Cresol from the Ether Fraction.—The ether extracts were freed from ether by distillation first at ordinary pressure and then *in vacuo*. There were thus obtained 5.5 gm. of pale brown oil having the odor that is characteristic of *p*-cresol. This oil distilled over completely at a temperature of 198–200° at

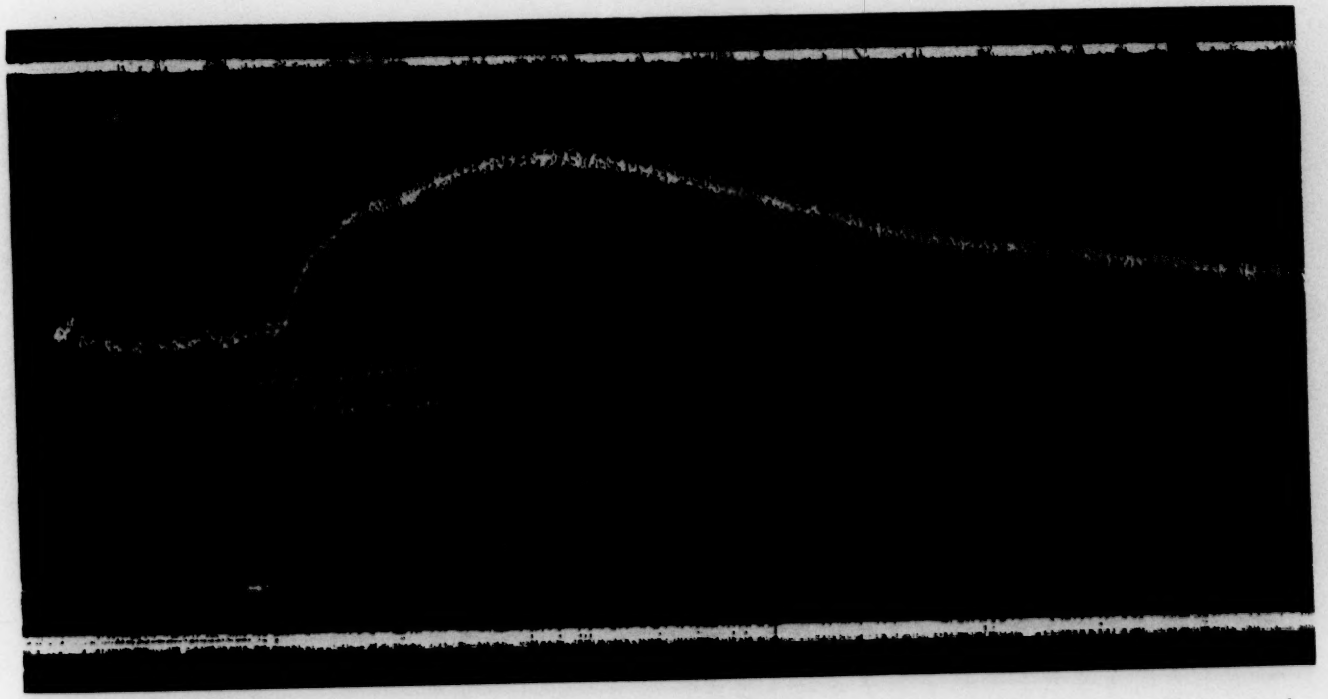

FIG. 1. Respiratory and blood pressure tracing obtained by injecting 2.0 mg. of synthetic tyramine hydrochloride into the femoral vein of a dog.

ordinary pressure and boiled at 90–91° at a pressure of 10 mm. The perfectly colorless oil obtained was pure *p*-cresol. (The recorded boiling point of *p*-cresol is 198°.)

Separation of Pure p-Hydroxyphenylacetic Acid.—The alkaline aqueous liquid was treated with an excess of hydrochloric acid and extracted with ether until the ether extracts no longer gave a Pauly reaction. The ether was then dried over calcium chloride and distilled at first under ordinary pressure and then *in vacuo*. There were thus obained 2.3 gm. of a pale yellow solid which was dissolved in water, the solution decolorized with charcoal, and redistilled *in vacuo*. The resulting white solid was dissolved in 4 cc. of hot water. 8 cc. of concentrated hydrochloric acid were then added drop by drop. This caused the *p*-hydroxyphenylacetic acid to precipitate in large, white, flat, prismatic needles melting sharply at 150° (Corrected). 1.7 gm. of the perfectly pure product was obtained. The recorded melting point of *p*-hydroxyphenylacetic acid is 148° (Uncorrected).

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